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Examining the role of genetic variability for drought stress responses in wheat (*Triticum aestivum* L.) and sorghum (*Sorghum bicolor* L. Moench.) and its implications for water and nitrogen use efficiency

Inaugural Dissertation for a Doctorate Degree

in Agricultural Science in the Faculty of Agricultural Science, Nutritional Science and Environmental Management

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“Usually, if you have tremendous pressure,

it's because an opportunity comes along”

Billie Jean King

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1. General introduction

1.1. Biology and domestication

1.1.1. Biology and domestication of wheat

The domestication of wheat marks a pivotal moment in human history, signifying the transition from a hunter-gatherer lifestyle to one based on agriculture (Preece et al., 2015). This transformation underscores the significance of wheat domestication as a key event in the emergence of complex societies (Kislev et al., 2004). Occurring in the region known as the "Fertile Crescent," encompassing the Mediterranean coast of Israel, Lebanon, Syria, south-eastern Turkey, northern Iraq, western Iran, and neighbouring regions such as Transcaucasia and northern Iran, wheat domestication is characterized by a complex evolutionary process (Matsuoka, 2011).

A major factor driving the evolution of *Triticum* species is allopolyploidization, resulting from hybridization with *Aegilops* species (Tsunewaki, 2009). Approximately one million years ago, diploid species *T. monococcum* and *T. uratu*, carrying the A genome, emerged (Huang et al., 2002). Half a million years later, tetraploid species *T. turgidum* (AABB genome) and *T. timopheevii* (AAGG genome) originated. Cultivated forms of these species, such as *T. turgidum* L. *ssp. dicoccon* and *T. timopheevii* Zhuk *ssp. timopheevii*, appeared alongside the development of agriculture (Shewry, 2009). Concurrently, *T. monococcum* diversified into its wild and cultivated forms, *T. monococcum* ssp. *monococcum* (Einkorn). Subsequently, natural hybridisation between tetraploid species and diploid wild grass *Aegilopsis tauschii* led to the evolution of hexaploid *Triticum* species. Bread wheat *T. aestivum* ssp. *aestivum* (AABBDD genome) emerged from the hybridization of *T. turgidum* with *Aegilops tauschii* (DD genome) (McFadden and Sears, 1946). Additionally, *T. zhukovskyi* (AAAAGG genome) arose from the hybridization of *T. timopheevii* and *T. monococcum* in the Transcaucasia region. Unlike their diploid and tetraploid counterparts, no wild forms of hexaploid wheat are known, suggesting inherent cultivation status (Dvorak et al., 1993). This evolutionary passage illustrates the coevolutionary interplay between natural processes and human activities that shaped the diversity of wheat species we recognize today. Thus, the hybridization and the permanent anthropogenic influence on the cultivated species have developed in the *Triticum* species, on which in particular the success of baking wheat (*Triticum aestivum* L. ssp. *aestivum*) is based.

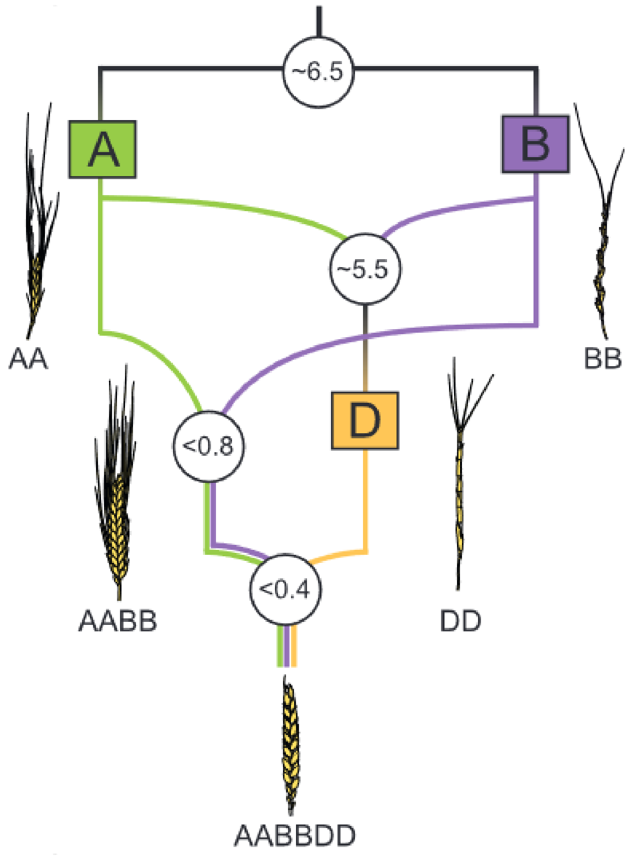

Figure 1: Evolution of bread wheat (*Triticum aestivum* L. ssp. *aestivum*). Phylogenetic model of allohexaploid wheat with approximate date for divergence and the three main hybridization events are given in white circles in units of million years (My). The differentiation of the wheat lineage (*Triticum* and *Aegilops*) from a common ancestor into the A and B genome lineages began approx. 6.5 My ago and is marked with the black coloured part of the phylogenetic three. The first hybridisation occurred before approx. 5.5 million years and let to the origin of the D genome lineage due to homoploid hybrid speciation. The formation of allotetraploid emmer wheat (*Triticum turgidum*; AABB) occurred through a second hybridization event between a close relative (BB) of *Aegilops speltoides* and *Triticum urartu* (AA), resulting in allopolyploidization. Subsequently, bread wheat originated from a third hybridization event, where emmer wheat and *Aegilops tauschii* (DD) combined, furthering the allopolyploidization process (Source Marcussen et al., 2014).

The non-brittleness of the spike and the possibility of free threshing are the most outstanding characteristics of domesticated wheat. The fully non-brittleness of the spike was achieved by an accumulation of mutations over time, according to the archaeologically documented gradual development of this trait (Kislev, 1984; Tanno and Willcox, 2006). The brittle rachis trait in ssp. *dicoccoides* is controlled by two major loci situated on the short arms of chromosomes 3A and 3B (Levy and Feldman, 1989). Avni et al. (2017) successfully identified these two genes, which are homologous to the barley Brittle Rachis genes *Btr1* and *Btr2* (Pourkheirandish et al., 2015). They demonstrated that while a wheat homolog of *Btr2* exists, it's the mutations in the *Btr1* orthologs within the wild emmer wheat loci (*br-A1* and *br-B1*) that lead to non-brittleness. The second crucial domestication trait, free threshing, emerged at the tetraploid level in the already domesticated non-fragile crop ssp. dicoccon. Muramatsu (1986) demonstrated that all free-threshing forms of tetraploid wheat carry a dominant allele, designated the Q factor, located on the long arm of chromosome 5A (Sears, 1954). The Q allele has pleiotropic effects, altering glume morphology, spike compactness, and other phenotypic characteristics (Mac Key, 2005; Simons et al., 2006).

1.1.2. Biology and domestication of sorghum

The domestication of Sorghum (*Sorghum bicolor* L. Moench) began in Northeast Africa, in present-day Ethiopia and Sudan, around 4000-3000 BC (Windpassinger et al., 2015). Disruptive selection practices led to the development of numerous improved Sorghum varieties, which spread through human migration and trade routes to other regions of Africa, India (around 1500-1000 BC), the Middle East (around 900-700 BC), and China (around 400 AD). Sorghum was brought to Europe around 1204 and to the American continent in the 1750s, initially as broomcorn and later as grain and sweet sorghum in the first half of the 19th century (Windpassinger et al., 2015). Adaptation to various environments and selection for different uses resulted in significant morphological and genetic diversity, which remains largely unexploited. The vegetative growth of Sorghum resembles that of maize, but unlike modern maize varieties, Sorghum exhibits varying degrees of tillering depending on genotype, environment, and their interaction. Although Sorghum is predominantly self-pollinating, it can experience up to 50% cross-pollination depending on panicle structure and weather conditions during flowering. Five morphological subspecies or races of Sorghum bicolor and their intermediates can be distinguished based on panicle structure, each adapted to different climatic conditions and agricultural requirements.

Sorghum exhibits significant variation in plant height, ranging from dwarf types of only 50 cm to tall biomass types of up to 5 m. Similar to maturity, plant height is controlled the presents/absents of four major dwarfing loci (*Dw1*, *Dw2*, *Dw3*, *Dw4*), with tallness being incompletely dominant (Quinby and Karper, 1945; Brown and Paterson, 2013). *Dw3* has been identified as an auxin transporter, with its recessive form being unstable and reverting to the dominant form causing tallness. While shorter genotypes do not necessarily affect the harvest index, taller genotypes generally boast a higher grain yield potential, likely due to increased availability of stem reserves, especially advantageous under stressful conditions. However, modern grain types are typically recessive at three of the four *Dw* loci, with a plant height not exceeding 170-180 cm to facilitate combine-harvesting. Extremely short 4-dwarfs are rarely utilized due to their notably low yield potential.

1.2. Relevance of grains for human nutrition

1.2.1. Contribution of wheat on human nutrition

Wheat (*Triticum aestivum* L. *ssp. aestivum*) is one of the three most relevant crops worldwide, contributing around 21% to the world's food supply. It is the primary crop for human nutrition in Europe and other temperate regions (Ortiz et al., 2008). Global wheat production reached an average production volume of 766.93 million metric tonnes over the period from 2018 to 2022. (Figure 1; FAOSTAT, 2024). According to the U.S. Department of Agriculture (USDA), the production was mainly contributed by the European Union (20.91%), China (17.42%), and India (12.17%). Over the period from 2018 to 2022, the worldwide average yield was 3.52 t/ha.

Globally, approximately 71% of wheat produced is utilized for human consumption, while 17% is used as animal feed. In certain regions as Europe, North America, and Oceania, the animal feed fraction can account for up to 25% of the regional production (Kim and Dale, 2004).

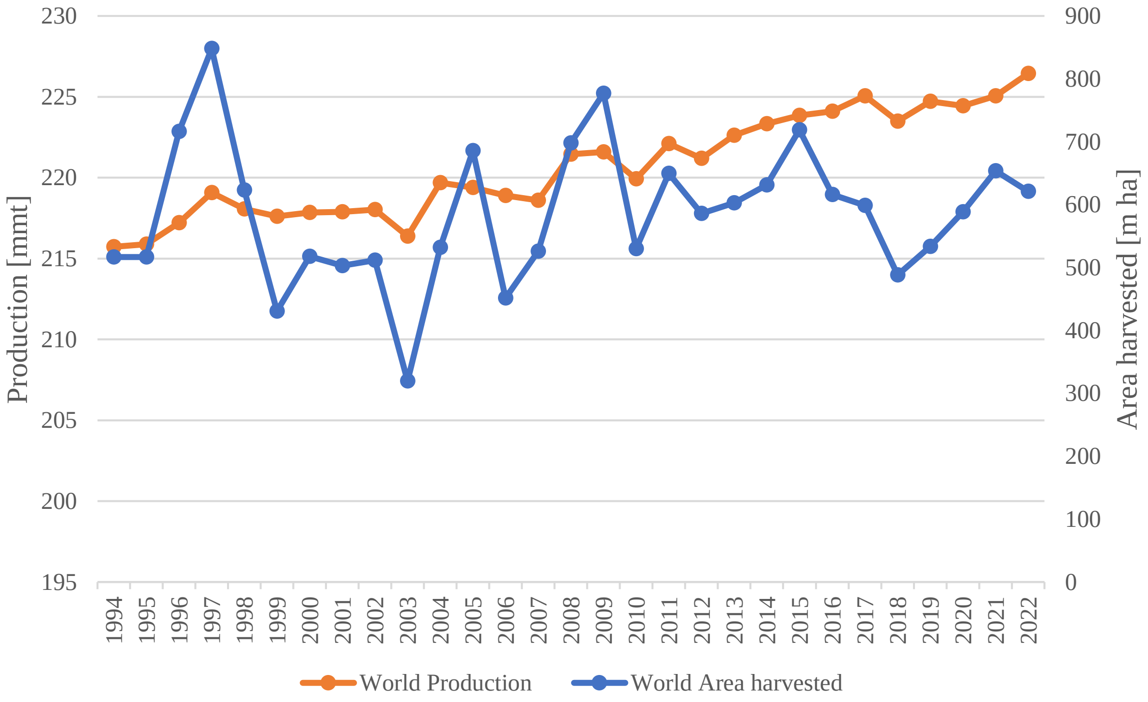

Figure 2: World production and global cropping area of bread wheat. Total grain yield of bread wheat in million metric tons [mmt] and production area harvested in million hectares [m ha] from 1994-2022.

It is predicted that the demand for major food crops will exceed the available supply by 2050, potentially resulting in approximately 318 million people (4% of the world population) experiencing hunger. However, it is important to exercise caution when interpreting these calculations, as they refer to the low food prices in 2005/2007 (Alexandratos and Bruinsma, 2012; Fischer et al., 2014; McKenzie and Williams, 2015). To address the disparity between production and consumption, several strategies can be considered. These include increasing crop production per farm, optimizing the use of resources such as water, nutrients, and energy, transitioning to a less meat-intensive diet, and reducing food waste (Foley et al., 2011).

1.2.2. Contribution of sorghum on human consumption

Sorghum (*Sorghum bicolor* L. Moench) is one of the world's most important cereals and ranked fifth in global production during the 2010 - 2020 period (FAO, 2023). Thanks to its versatility and tolerance to various abiotic stressors, Sorghum is of high importance for subsistence farming, due to its good performance under low-input conditions in Africa and Asia, especially in India. Global sorghum production reached 57 million metric tons in 2022 (Figure 1; FAOSTAT, 2024). However, the United States was the largest producer during this period, with the top ten producers worldwide accounting for 76% of global production. In general, two groups of countries can be distinguished that grow Sorghum. Commercially oriented countries like the USA, Mexico, Argentina, Australia, and China primarily grow Sorghum for animal feed, export and biofuel production. Thanks to hybrid seeds and modern agricultural practices, these countries achieve relatively high grain yields averaging four tons per hectare.

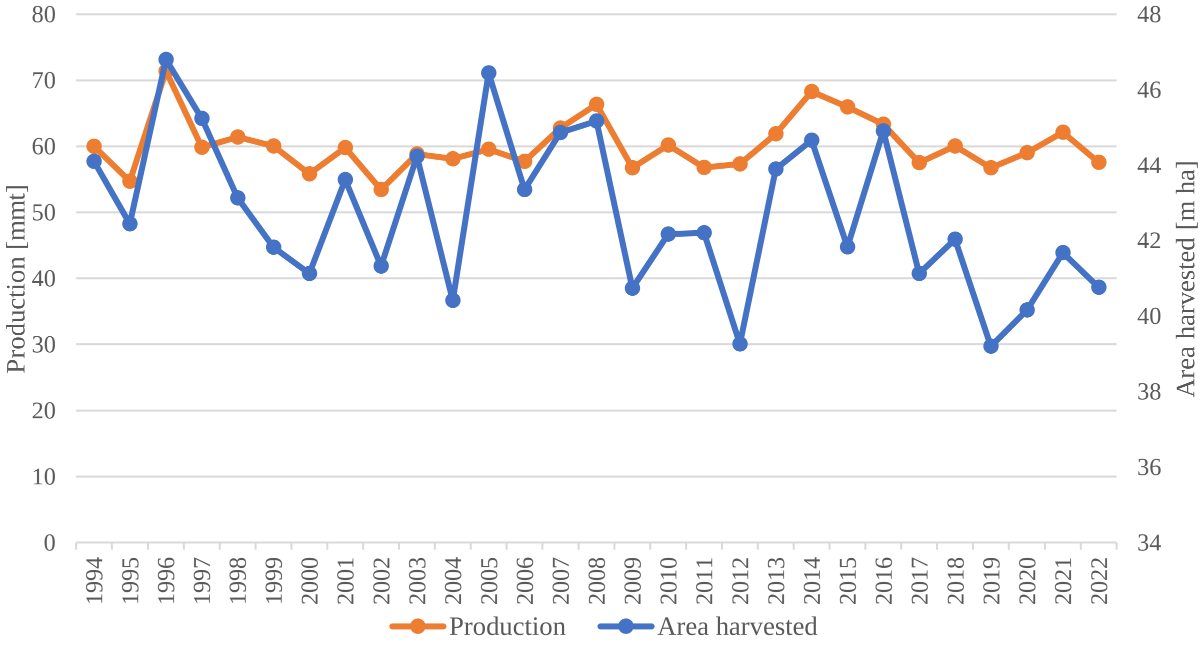

Figure 3: World production and global cropping area of sorghum. Total grain yield of Sorghum in million metric tons [mmt] and production area harvested in million hectares [m ha] from 1994-2022.

In contrast, countries like India, Senegal, Sudan, and other African nations, where traditional low-input farming systems make the majority of agricultural food production, Sorghum is primarily grown for human consumption, with average yields as low as 1 ton per hectare (Regassa and Wortmann, 2014). Large-scale cultivation for forage and silage is common in Argentina, Mexico, and the USA, which collectively account for over 80% of global production for this purpose (Burks et al., 2015). The cultivation of sweet Sorghum enables sugar or ethanol production in tropical and subtropical regions unsuitable for sugarcane due to either dryness or cold winters. Especially in the USA and China, this is gaining interest in production (Burks et al., 2015; Mocoeur et al., 2015). Additionally, biomass or fibre Sorghum is used for cellulose production and bioenergy generation (Zegada-Lizarazu and Monti, 2012).

1.3. Drought: A major threat for global food production

1.3.1. What is drought?

Drought is a recurring and extreme climatic event that occurs on a land mass and is characterised by below-average precipitation (Dai, 2011). In most cases, the classification of the amount of precipitation refers to a period that can extend from months to years. As drought events are always considered in relation to an assumed regional normal value, droughts can also occur in regions that are generally exposed to high levels of precipitation and are therefore a globally occurring weather phenomenon (Dai, 2011). Drought stress-causing events can be divided into three groups: meteorological drought, agricultural drought and hydrological drought (Khadka et al., 2020). Meteorological drought is defined as a period of below-average precipitation, typically spanning months or years, accompanied by above-average temperatures (Gianni et al., 2003; Langridge and Reynolds, 2020).

A meteorological drought is often the initial trigger for other, more specific or regional drought events, and is also favoured in its development by local conditions, such as reduced evaporation from dry soils or high temperatures (Dai, 2011). In contrast, agricultural drought is defined as a period in which the soils used for production have below-average moisture (Spinoni et al. 2019; Langridge and Reynolds, 2020).These can occur due to a reduction in precipitation, an increase in precipitation intensity but a reduction in the number of precipitation events, or an increase in evaporation rates (Schubert et al., 2004; Dai, 2011). In contrast to the other two forms of drought, hydrological drought occurs with a delay and arises when water reservoirs such as lakes, rivers or artificial catchment facilities fall below the long-term average (Dai, 2011; Khadka et al., 2020). Furthermore, drought stress causing events can be categorised into mild, moderate or severe events according to the duration and amount of unavailable water for plants (Khadka et al., 2020). It is important to note that there is no clear classification when categorising a drought, due to the number and severity of distorting factors. These include, but are not limited to, a lack of precipitation, soil composition, soil type, susceptibility of the production system and the duration and level of temperature increases (Spinoni et al., 2019; Khadka et al., 2020). Other environmental factors that can influence plant growth and development include soil salinization, acidification and the formation of toxic amounts of aluminium or boron. These factors can lead to reduced root growth, which in turn can reduce the plant's drought stress tolerance (Rashid and Ryan et al., 2004; Blum, 2011; Langridge and Reynolds, 2020). In addition to environmental factors, the specific crop and the growth stage during which a drought event occurs have a significant influence on the intensity and impact of drought on production (Khadka et al., 2020; Langridge and Reynolds, 2020). Particularly vulnerable growth stages are emergence and early developmental stages as well as flowering time, which lead to lower biomass and lower yield potential during drought or heat events (Ottman et al., 2012).

Consequently, the parameters employed to characterise or quantify a drought event are of particular significance. From a meteorological perspective, droughts can typically be described by the three principal factors of intensity, duration and area coverage (Dai, 2011). An agronomic assessment of drought can be conducted by quantifying the growth of a crop, dry matter production or yield potential (Anjum et al., 2011; Zhang et al., 2018). In particular, yield is used in breeding programmes to validate drought stress resistance (Zhang et al., 2006). However, depending on the onset, vigour and other prevailing environmental conditions, different genes are involved in the drought stress response, each contributing in small proportions to the expression of the trait (Sallam et al., 2019). For this reason, an understanding of the specific morphological, phenological or physiological response trough the plant in different environmental conditions is crucial to develop plants with an increased tolerance to drought stress.

1.3.2. Selection of traits to increase drought stress resistance

1.3.2.1. Above ground traits associated with drought stress

Climate change is leading to an increase in hardly predictable dry periods in many growing regions on the other (Tovignan et al., 2023). A major challenge when using physiological traits in plant breeding is the sensitivity of these traits to environmental changes (Khadka et al., 2020). In wheat, this is particularly the case with growing erratic drought stress events. Therefore, with regard to drought stress tolerance, the development of varieties that offer high yield potential and maintain to achieve high yields under both drought stress and favourable growing conditions is particularly important (Langridge and Reynolds, 2021). Sorghum is a crop that is well suited to environments with limited water availability and is often used as a model species to study drought tolerance (Dugas et al., 2011). Sorghum varieties in drought-prone environments have more pronounced water-saving mechanisms such as dense, extensive root systems to facilitate survival in times of water scarcity. Drought-tolerant and drought-resistant sorghum varieties have higher root weight, root volume and root-to-shoot ratio than their drought-susceptible counterparts (Nour and Weibel, 1978). Nevertheless, many studies have focussed on improving drought stress tolerance in sorghum (Kapanigowda et al. 2013; Sehgal et al. 2018; Bobade et al. 2019; Queiroz et al. 2019).

The stay-green trait is considered an example of the drought tolerance strategy. Stay green varieties maintain the chlorophyll content and photosynthetic activity of the leaves for longer despite drought stress conditions. Thus, this trait has been linked to improved yield stability and is therefore a potential target trait for cultivar improvement (Borrell et al., 2000; Harris et al., 2007; Jordan et al., 2012; Borrell et al., 2014; Christopher et al., 2016). The stay-green trait describes a change in the process of senescence in which the continuous degradation of chlorophyll and a gradual reduction in photosynthetic activity takes place (Xu et al., 2000). Crop varieties can be labelled as ‘functionally remaining green’ if the overall senescence processes are delayed or the rate of progression is reduced while photosynthesis continues (Thomas and Howarth, 2000; Borrell et al., 2014). This differs from ‘cosmetically green remaining’ phenotypes, where only the process of chlorophyll degradation is delayed or slowed down (Thomas and Howarth, 2000; Thomas and Ougham, 2014). In Sorghum, the introduction of stay-green Quantitative Trait Loci (QTL) into a senescent sorghum background has been observed to reduce tillering and upper leaf size, resulting in a smaller canopy and decreased water usage during anthesis (Borrell et al., 2014). This leads to pre-anthesis water conservation, which can be used post-anthesis to improve grain yield and size. These findings establish a connection between the role of stay-green QTL in modifying canopy development and the resultant impact on crop water utilization patterns and grain yield under terminal drought conditions.

Early vigour (EV), defined as the rapid expansion of leaf area during the initial growth phases of plants, is recognized as a trait conducive to drought tolerance (López-Castañeda, 1995; Blum, 2005). It has been observed in previous studies that swift leaf area development in the early stages of plant growth results in increased shading of the soil surface. This shading reduces soil radiation and decreases evaporative water loss, allowing the plant to make better use of available water later in the growing season (Siddique et al., 1990).

Accumulated data from various wheat and barley studies indicate that when combined with strong stand establishment, EV optimizes water use efficiency (WUE) and improves the transpiration-evapotranspiration ratio in crops (Richards et al., 2002; Condon et al., 2004). However, the advantages and disadvantages of EV are closely linked to its impact on water stress patterns throughout the crop growth cycle. While EV can enhance light interception and photosynthesis, reduce soil evaporation, and improve the crop's competitiveness against weeds, it may also increase water and nutrient uptake before flowering, potentially leading to water and nutrient scarcity during grain filling (Condon et al., 2004; Hammer et al., 2016). In addition to its benefits in drought resistance, EV has been shown to positively affect nitrogen and phosphorus uptake and acquisition efficiency in wheat, which is particularly advantageous in phosphorus-limited environments (Liao et al., 2006; Ryan et al., 2015). Moreover, EV is associated with increased competitiveness against weeds and enhanced root growth (Bertholdsson, 2005). Given the rising herbicide resistance of weeds, the weed competitiveness conferred by EV presents a viable alternative. Investigations across multiple crops have produced promising findings in this regard (Dingkuhn et al., 1999; Coleman et al., 2001; Bertholdsson, 2005).

López-Castañeda et al. (1995) conducted a comparative study of several cereal crops (barley, bread wheat, durum wheat, triticale, and oats) regarding EV and found barley to exhibit the highest vigour. They noted that embryo size accounted for approximately 90% of the variation in vigour between species. Furthermore, the study suggested leaf width as a reliable indirect measurement of embryo size and specific leaf area. Subsequent research has identified key physiological traits associated with EV, including seed mass, embryo size, leaf width, leaf area, and coleoptile tiller production (Rebetzke and Richards, 1999; Maydup et al., 2012; Moore and Rebetzke, 2015).

Studies on the genetic regulation of EV have revealed a significant relationship between reduced EV in wheat and gibberellic acid (GA)-intensive dwarfing genes, which were introduced into modern cultivars during the Green Revolution. Examples of such GA-intensive genes include *Rht-B1b* and *Rht-D1b* (Coleman et al., 2001; Rebetzke et al., 2007). Regarding root characteristics, Maydup et al. (2012) found that increased root biomass does not negatively affect EV. Conversely, Li et al. (2017) suggest a pleiotropic effect associated with *Rht* genes, as they observed reduced root spreading. Ellis et al. (2004) identified alternative *Rht* genes in wheat (*Rht* 8 and *Rht* 12), characterized as GA-insensitive, which do not reduce coleoptile length and leaf elongation while still reducing plant height. These alternative *Rht* genes express at later stages of development, offering a means to introduce EV in modern wheat cultivars.

Since sorghum is predominantly grown in arid areas with shallow soils and low water capacity, it is particular prone to drought stress during early developmental stages, which lead to significant yield losses (Queiroz et al. 2019; Tovignan et al., 2023). Under combined drought and heat stress conditions during seedling emergence and establishment, seedling death is a common problem. Ndlovu et al (2021) have shown that this is due to a reduction in photosynthesis and chlorophyll content. In sorghum, drought-induced stand losses can occur after full emergence and before seedlings are established (Queiroz et al. 2019). The early stage of plant growth, namely germination, emergence and seedling establishment, is potentially the most susceptible to drought stress. For this reason, much attention has been paid to the effects of drought-induced water deficit on the early developmental stages of sorghum. However, there are significant differences between sorghum genotypes in terms of their response to different degrees of drought stress, translocation of photo assimilates and nutrient uptake from soil. These differences result in lower grain yield and quality (Assefa et al., 2010; Kapanigowda et al., 2013; Sehgal et al., 2018). According to Assefa et al. (2010), drought stress at the vegetative stage can reduce yield by more than 36%. Therefore, it is important to develop effective methods to reduce yield losses due to post-flowering drought-stress as well as effective mechanisms to reduce yield losses due to pre-flowering drought-stress. This could also reduce the effects of post-flowering stress, which is particularly intense and significant when drought stress prevailed during the vegetative phase (Crafurd and Peacock, 1993).

1.3.2.2. Below ground traits associated with drought stress

Roots, as the primary organ of nutrient and water uptake, are of particular significance to increase drought stress resistance as well as to improving crop yield stability particularly in marginal growing regions (El Hassouni et al., 2018; Voss-Fels et al., 2018a; Voss-Fels et al., 2018b; Alahmad et al., 2019). The temporal and spatial distribution of the root system significantly influences the source-sink relationship, directly impacting the agronomic performance of crops under both optimal and stress conditions (Uga et al., 2013). Besides providing adequate water and nutrient supply to the above-ground organs, roots play a pivotal role in physically anchoring the plant, especially under heavy spikes (Lynch, 2013). A larger root mass offers environmental benefits by enhancing soil organic matter content, thereby improving soil fertility and structure (Dupuy et al., 2010), consequently contributing to better erosion control (Kumar et al., 2006). However, the value of a large root system must always be seen in the context of the above-ground biomass, as both components of the biomass structure compete for nutrients and only an optimal root-shoot ratio is beneficial for the agronomic performance of the plant (Plata et al., 2011; Voss-Fels et al., 2018b). Despite their importance, roots had received relatively little attention in research for a long time. Methodological challenges, time constraints, and cost considerations have hindered extensive root studies. Moreover, the complexity of soil conditions, which often exhibit variations at a small scale, poses difficulties for further root investigations. Many root studies have presumed homogeneous soil conditions, potentially deviating from natural conditions (Lynch, 1995; Pierret, 2008). Roots exhibit dynamic responses to their growth medium and are distributed heterogeneously within it (Atkinson, 2000). While mutations in certain genes have been demonstrated to influence root traits such as primary root length, root branching, and root hair formation, our understanding of these genes and their mechanistic implications for phenotype remains limited (Kell, 2011). Nonetheless, substantial progress in identifying major genes controlling root architecture in most crop species, in comparison with above-ground traits, has been slow. An exception to this trend is rice, where the first cloned major gene, *DEEP ROOTING 1* (*DRO1*), has been found to confer a yield advantage under water-limited conditions (Uga et al., 2013). Recently discovered genes, including *QUICK ROOTING 1* and *2*, have been shown to enhance root length in rice. These genes hold the potential to be combined with *DRO1* to develop a variety of root ideotypes, each tailored to a specific target environment (Kitomi et al., 2018). Identifying gene targets for genetic manipulation of roots presents opportunities to directly and precisely modulate root characteristics. However, it remains uncertain which type of root system is most advantageous under specific environmental conditions.

According to Lynch (2013) a root system which follows the principle of being 'steep, cheap, and deep' is beneficial for most crops cultivated in water-limited environments with deep soils. In contrast, in higher rainfall environments or shallow soils subject to sporadic rainfall during the growing season, an extensive, shallow, and broad root system is typically favoured

(Figure 4). These examples underscore the significant context-dependency inherent in optimizing root architecture according to environmental conditions. Furthermore, the effects of above-ground development, including the duration of the vegetative period and the timing of flowering, exert a confounding influence on nutrient and water demand. Hence, investigating the interactions between flowering time and specific root ideotypes is crucial. To address this question and delineate breeding targets, it is imperative to develop test lines with similar flowering times but differing root ideotypes.

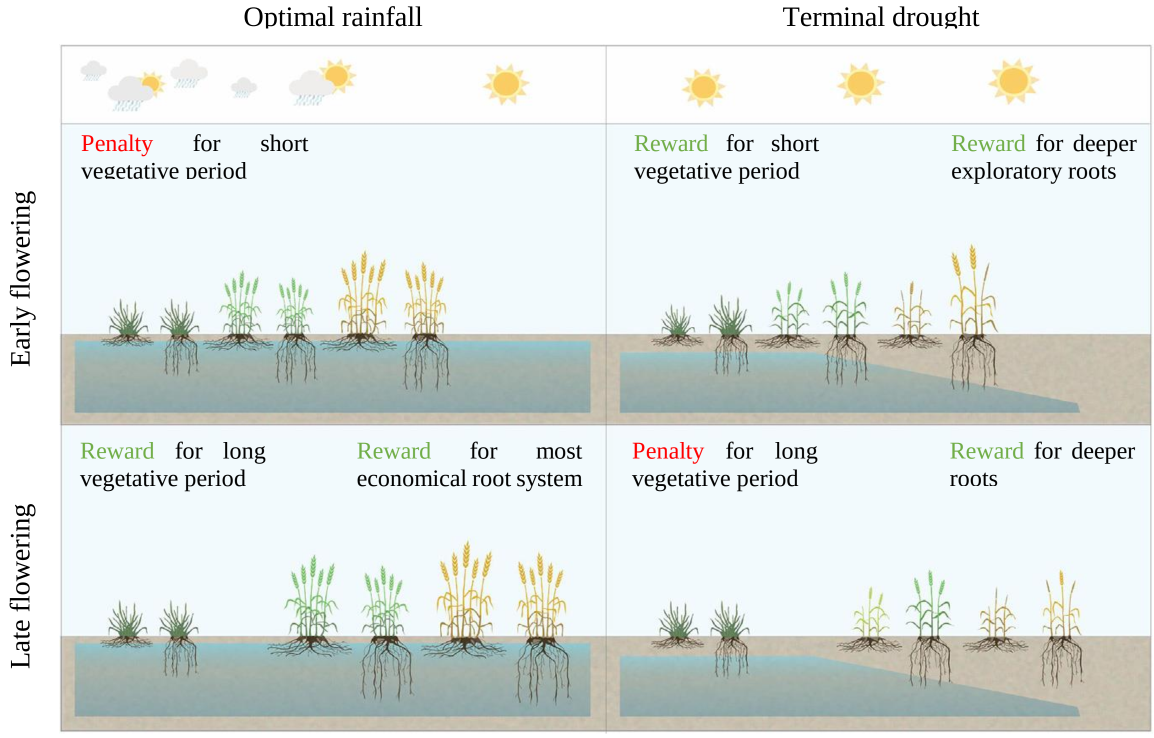

Figure 4: Rewards and penalties associated with root architecture in the context of water availability and flowering time (Source: Voss-Fels et al., 2018b).

1.3.3. Use of modern tools to increase drought stress resistance

1.3.3.1. Molecular genetic approaches in plant breeding

Modern high-throughput genotyping methods, provide a cost-effective technique for rapidly characterising thousands of single nucleotide polymorphisms (SNPs), which can be utilised to identify trait-associated regions (Varshney et al., 2014). In order to link complex traits such as abiotic stress tolerances with specific genomic regions at high resolution, different approaches have been developed, including biparental populations or genome-wide association studies (GWAS) (Price et al., 2006; Jordan et al., 2012). GWAS is various times successfully used to identify complex traits in wheat (Zegeye et al., 2014; Mwadzingeni et al., 2017; Nouraei et al., 2024) and Sorghum (Morris et al., 2013; Wang et al., 2020; Ortiz and Salas-Fernandez, 2022; Xin et al., 2022). However, GWAS is mostly used to detect minor QTL effects of a certain trait. Subsequently, near-isogenic lines (NIL) can been used to validate certain QTL due to their low background noise (Prasad et al., 2003; Reynolds and Tuberosa, 2008; Mwadzingeni et al., 2017). The successful application of marker-assisted selection (MAS) to produce sufficient NILs is governed by factors like the number of targeted QTLs, the resolution of the marker map and the crossing scheme (Frisch and Melchinger, 2005). Despite modern marker technology offers potentially genetic maps of higher resolution at low costs, the difficulty of an insufficient population structure needs to be overcome. Thus, different approaches have been made such as nested-association mapping (NAM) populations (Marla et al., 2019) or back-cross-NAM (BCNAM) (Jordan et al., 2012) as well as diversity panels have been successfully applied to identify genomic regions of interest. Diversity panels are especially of interest for drought stress resistance advantageous, since, unlike for grain yield, most conventionally used elite varieties were not selected based on drought related physiological traits and hence offer a large genetic variation on traits such as radiation efficiency, biomass, heat adaptation or root system characteristics (Dreccer et al., 2012; Langridge and Reynolds, 2020; Molero and Reynolds, 2020). An alternative resource to increase the genetic variance within a population is the use of land races which are available in many collections worldwide, offer an immense opportunity to tap new sources for drought stress resistance and are relatively easy to incorporate into populations due to good crossing ability (Ogbonnaya et al., 2007; Trethowan and Mijeeb-Kazi, 2008; Dempewolf et al., 2014).

1.3.3.2. Precision Phenotyping Techniques for Drought Stress Resistance

Similar to the advances in genotyping, which have besides high-throughput genotyping via SNP-Chips dramatically reduced the cost and time of sequencing, there have also been tremendous advances in phenotyping (Rasheed et al., 2017; Jang et al., 2020). Particularly, for research and breeding under controlled environments, the use of high-precision phenotyping platforms is increasing, especially for in depth trait characterisation and dynamics (Kim et al., 2020). For field-based phenotyping, the use of sensor-based systems combined with unmanned aerial vehicle (UAV) such as drones or autonomous ground-based robots is becoming increasingly prevalent (Bangert et al., 2013; Grimstad et al., 2015; Zhang et al., 2021; Guo et al., 2021). In particular, the utilisation of drone-based sensors in commercial breeding programmes demonstrates the efficacy of sensor-based phenotyping for the characterisation of traits and trait-environment dynamics (Haghighattalab et al., 2016; de Oliveira et al., 2021). Specifically, growth indices such as normalised difference vegetation index (NDVI), green normalised difference vegetation index (GNDVI) and normalised difference red edge index (NDRE) need to be mentioned, since they allow an accurate determination of emergence, canopy and senescence under diverse environmental conditions and throughout the entire life cycle (Jang et al., 2020; Prey, et al., 2022).

Similarly, as a consequence of the technical progress that has facilitated the implementation of new phenotyping methods in the field through the use of sensor-supported UAV’s, there has also been a notable advancement in phenotyping under controlled conditions. For instance, novel digital image-based methodologies enable the examination of seed and embryo size, leaf pigmentation, biomass, or fruit development under both favourable and abiotic stress conditions (Perez-Sanz et al., 2017; Tardieu et al., 2017; Anupama et al., 2018; Spindel et al., 2018). For instance, image analyses have demonstrated that sorghum plants exhibiting water and nitrogen deficits on their stems demonstrate distinct phenotypic characteristics (Neilson et al., 2015). In addition to imaging methods, gravimetric measurement of transpiration is a widely used system, particularly for the individual investigation of genotypes for their water consumption and drought stress tolerance under different simulated environmental conditions (Dixon et al., 2023). The gravimetric systems postulate on the assumption that genotypes adopt different strategies with regard to water requirements. Some genotypes can reach deeper soil layers via pronounced root systems, while other genotypes exhibit conservative water consumption depending on the stage of development (e.g. pre-anthesis) (Passioura, 2006; Saradadevi et al., 2017). Thus, the plant’s responses to water uptake and release are reflected in a specific transpiration pattern, which can be measured gravimetrically (Saradadevi et al., 2017; Dixon et al., 2023). Unlike above ground traits, the phenotyping of root characteristics under field conditions presents significant challenges. Consequently, phenotyping methods have been devised and tailored to artificial growth settings, strategically targeting components of the root system that are readily measurable. These systems typically comprise multiple soil-filled root chambers, facilitating non-destructive phenotyping (Johsi et al., 2017). Among the common methods of root phenotyping is the rhizobox system, which, when coupled with spectral image analysis, offers a means of acquiring root architecture parameters (Nakaji et al., 2008; Kusumo et al., 2011). Other phenotyping approaches utilize high-resolution cameras to capture 3D images of root system architecture (Clark et al., 2011), or employ magnetic resonance imaging to track root development and water transport (Van Duschoten et al., 2016). A comprehensive understanding of the genetic architectures and regulatory mechanisms governing root system characteristics is crucial for achieving optimal agronomic performance and productivity. Root system architecture is predominantly under genetic control (Kato et al., 2006; Lucas et al., 2011), and thus merits primary attention. Numerous genes have been identified to enhance both root architecture and plant yield (Tuberosa et al., 2002; Steele et al., 2006; Hund et al., 2009).

1.4. Nitrogen use efficiency in the context of wheat

1.4.1. Agroecological context of nitrogen

Nitrogen (N) is the primary plant nutrient that has to be fertilized in the highest quantities in wheat cultivation, in order to achieve the qualitative and quantitative objectives (Swarbeck et al., 2019). To ensure sufficient protein production, farmers typically apply three doses of nitrogen (Hawkesford et al., 2017). The artificial synthesis of ammonia demands a high consumption of fossil fuels, which in turn releases a tremendous amount of CO_2 and accounts for about 2% of global energy demand (Sutton et al., 2013; Humphreys et al., 2021). Further, the excessive use of nitrogen fertilizers can have adverse environmental impacts, to the extent that it has been suggested that a safe planetary boundary may have been exceeded (Zhang et al., 2015; Schulte-Uebbing et al., 2022). Despite exceeding a global limit, which was described by Rockström et al. (2009) and predicted by Steffen et al. (2015) at a safe level of half the currently used N, there are various studies that call for regional shifts while respecting the global limit values. The proposed strategy is to reduce the use of fertilisers, in countries where the intensive use of fertilisers is prevalent in agricultural practices, in order to address supply gaps in areas with more extensive cultivation systems. This approach is expected to enhance yield potential globally, while the global boundary is not exceeded (Nykvist et al., 2013; Heistermann et al., 2017; EEA, 2020). Nonetheless, in agricultural ecosystems, direct emissions of ammonia and nitrous oxide make a significant contribution to climate change, while nitrate leaching heavily contributes to groundwater pollution (Galloway et al., 2008; Keeler et al., 2012; Gao et al., 2023). Due to these challenges, there is an increasing interest in breeding varieties that exhibit higher nitrogen use efficiency (NUE) to achieve the required yield and quality parameters while reducing the ecological footprint (Lupini et al., 2020). The definition of NUE is grain yield or biomass production per unit of available soil N. However, the estimation of NUE can be approached by various approaches, thus the specific definition for deriving NUE can vary depending on scientific inquiries, cultivation conditions, and measurement methodology (refer to Table 1). Further, NUE can be calculated as the product of nitrogen uptake efficiency (NupE) and nitrogen utilisation efficiency (NutE) (Moll et al., 1982; Bouchet et al., 2016). NupE refers to a plant's ability to mobilise and acquire nitrogen from the soil, while NutE refers to a plant's ability to transport the absorbed nitrogen into the yield-determining organs (Moll et al., 1982; Good et al., 2004; Bouchet et al., 2016). Depending on the availability of nitrogen and other environmental conditions, such as water availability, NutE and NupE are weighted differently in achieving high NUE.

Table 1 Definitions and formulae to describe nitrogen use efficiency in plant (Source: Table in modified form from Good (2004)).

Equation Name	**Formula**	**Definition**	**Reference**
Nitrogen Use Efficiency	NUE = Sw ÷ N	Sw, shoot weight (DW); N, nitrogen content of shoots (DW)	Steenbjerg and Jakobsen, 1963
Usage Index	UI = Sw × (Sw ÷ N)	Sw, shoot weight; N, nitrogen in shoots	Siddiqi and Glass, 1981
Nitrogen Use Efficiency (Grain)	NUE = Gw ÷ N	Gw, grain weight; Ns, nitrogen supply (g per plant)	Moll et al., 1982
Uptake Efficiency	NupE = Nt ÷ Ns	Nt, total nitrogen in plant; Ns, nitrogen supply (g per plant)	Moll et al., 1982
Utilisation Efficiency	NutE = Gw ÷ Nt	Gw, grain weight; Nt, total nitrogen in plant	Moll et al., 1982
Agronomic Efficiency	AE = $(Gw_F - Gw_c) \div N_F$	N_F, nitrogen fertilizer applied; Gw_F, grain weight with fertilizer; Gw_C, grain weight of unfertilized control	Craswell and Godwin, 1984
Apparent Nitrogen Recovery	AR = (N_F uptake – N_C uptake) ÷N_F × 100	N_F uptake= plant nitrogen (fertilizer); N_C uptake= plant nitrogen (no fertilizer); N_F= Nitrogen fertilizer applied	Craswell and Godwin, 1984
Physiological Efficiency	PE = $(Gw_F - Gw_c)$ ÷ (N_F uptake – N_C uptake)	Gw_F, grain weight (fertilizer); Gw_C, grain weight (no fertilizer)	Craswell and Godwin, 1984

1.4.2. Nitrogen uptake

As mentioned in the previous chapter, the interaction between NupE and NutE and the respective parameters with the prevailing environment is a critical factor for NUE. Several studies have already identified traits to improve NUE, but without fully understanding their interactions with the environment or the interactions in the plant (Hirel et al., 2011). The reason for this is that the uptake of nutrients as a whole and the uptake of nitrogen in particular depends, on the one hand, on "external" factors and, on the other, on the plant's ability to absorb nutrients. With regard to the external factors, the availability of plant-usable N should be mentioned here, which is particularly dependent on the presence of nitrate (NO_3^-), as this is the main form of N uptake (Wolt, 1994). In addition to the availability of N, the volume available for rooting and the hydrology of the soil are also important external factors. While the external factors can be particularly influenced by arable farming measures, the ability to absorb N at plant level is particularly suitable for increasing NUE by breeding means and thus creating ecologically more favourable conditions at arable farming level. The ability to take up N can be considered at the cellular level, where nitrate and ammonium transporters enable the transfer of N to the intracellular region (Xu et al., 2012). Furthermore, N uptake can be considered at the morphological level. Since the root is the primary organ for N uptake, many studies on increasing NupE focus on this organ. A particular focus here is an increased root length and increased root density over the entire horizon as potential breeding traits (Foulkes et al., 2009, Duncan et al., 2017). Carvalho and Foulkes (2011) also state that a deep-reaching root system that is homogeneous over the entire horizon could act as an ideal type for increased N uptake from deeper soil layers. In addition to root traits, another breeding trait that has a positive effect on NupE is an increase in N accumulation in the vegetative organs, such as leaves and stems (Foulkes et al., 2009; Gaju et al., 2014).

Several studies have focused on the description of the genetic control of the root system and root morphology as well as the characterisation of candidate genes (O'Toole and Bland, 1987; Hoad et al, 2001; Ehdaie and Waines, 2003; Ford et al, 2006; Voss-Fels et al, 2016; El Hassouni et al, 2018; Alahmad et al, 2019). Despite the characterisation of more diverse candidate genes for genetic adaptation of the root system to increase NUE, practical applications are difficult because there is a large variation in the expression of the traits due to soil properties, nutrient and water availability and the ability to sufficiently penetrate the soil (Cormier et al., 2016).

1.4.3. Nitrogen utilisation

From an agronomic perspective, NutE refers to the effective utilisation per unit of nitrogen absorbed in the development of yield-forming organs (Gaju et al., 2014). N remobilisation from the source to the sink is a key parameter for increasing NutE and is reflected in the nitrogen harvest index. Several studies have investigated N assimilation at the molecular level to improve utilisation through optimised gene expression (Dubois et al., 2003; Boisson et al., 2005; Kaiser et al., 2011). Boisson et al. (2005) categorised the genes involved in N assimilation into three main groups: those encoding nitrate reductase, nitrite reductase, and the genes for the formation of glutamate reductase. After N is taken up by the roots in the form of NO_3^-, it is first reduced to nitrite (NO_2^-) by nitrate reductase (Kaiser et al., 2011). The process begins with the reduction of NO_2^- to ammonium (NH_4^+) through the use of nitrite reductase (Boisson et al., 2005). Subsequently, the incorporation into the amino acid glutamate occurs, first via glutamine synthetase using the intermediate glutamine, and finally through the use of glutamate reductase (Suzuki and Knaff, 2005). In addition to improving the understanding of N assimilation at the enzymatic level, the storage sites in the context of the plant's physiological development is relevant. Mesophyll cells contain up to 75% of the N bound in the leaves and are involved in photosynthetic processes through the enzyme Rubisco located in the chloroplasts (Monneveux et al., 2007). It can be inferred that a lack of nitrogen availability leads to a decrease in photosynthetic performance (Sylvester-Bradley et al., 1990; Monneveux et al., 2007). Foulkes et al. (2009) state that nitrogen deficiency results in a reduction in leaf surface area or radiation utilization, ultimately leading to a decrease in yield. Modern wheat lines achieve adequate soil coverage during anthesis, ensuring sufficient radiation utilization (Moreau et al., 2012; Gaju et al., 2014). Therefore, the most efficient approach to enhancing NutE through breeding methods is to boost N assimilation before anthesis (Shearman et al., 2005; Moreau et al., 2012). On marginal sites, increasing the specific leaf area per unit of N per leaf (SLN) can lead to an increase in N assimilation before anthesis and, consequently, an increase in NutE (Cormier et al., 2016). Furthermore, the expression of SLN is associated with easily quantifiable traits such as embryo size (López-Castañeda et al., 1996) and EV (Rebetzke and Richards, 1999).

Effective N remobilisation during the post-anthesis phase offers high potential to positively influence NutE and grain yield (Cormier et al., 2016). At anthesis, 35-45% of total N in wheat is in above-ground plant material, with the leaf and stem being the two most significant N stores (Barraclough et al., 2014; Gaju et al., 2014). However, although the leaf tissue achieves high remobilisation rates of 60-70%, the stem only records relatively low values of 48%. A high capacity for nitrogen uptake in the stem before flowering could theoretically result in a higher maximum nitrogen translocation and, consequently, a higher NutE (Foulkes et al., 2009). Furthermore, an increased ability to store nitrogen in non-photosynthetic organs could be advantageous in transferring a greater amount of nitrogen to the grains without compromising the plant's photosynthetic capacity (Bertheloot et al., 2008). However, the function for the significant nitrogen storage in non-photosynthetic organs remains unclear. Genetic variation in stem nitrogen content at anthesis has been reported in wheat (Pask, 2009; Barraclough et al., 2014; Gaju et al., 2014), as well as in nitrogen remobilisation. A correlation between post-anthesis nitrogen remobilisation efficiency and the onset of the rapid crown senescence phase was observed among 16 wheat cultivars grown at sites in the UK and France (Gaju et al., 2014). The transcription factor NAM-B1 has been found to accelerate senescence and increase nitrogen remobilisation from leaves to grains in wheat (Uauy et al., 2006). These results indicate that a better understanding of the mechanisms regulating nitrogen remobilisation after anthesis and senescence, in conjunction with environmental characterisation, particularly on nitrogen availability during the post-anthesis period, could provide the opportunity to increase grain yield and/or grain protein content in wheat cultivars.

1.5. Aim and objectives

Climate change will present significant challenges, particularly for the agricultural sector, as abiotic influences such as drought stress will be a limiting factor in non-irrigated production systems. From this perspective, there is a great interest among plant breeding in developing crops that can achieve high yields under less favourable growing conditions. Consequently, the identification of traits associated with higher water use efficiency has been a growing interest, such as EV which can reduce evaporation by rapid closure of the canopy or root morphology traits which can improve the exploitation of water resources and nutrient acquisition. For this purpose, a comprehensive knowledge of the phenological and morphological response of drought stress relevant traits as well as their genetic components affecting these traits is necessary. The work presented in this manuscript offers a combination of innovative phenotyping methods for two important crops, including gravimetric and image-based phenotyping, high-throughput field growth assessment, and analysis of nitrogen uptake and translocation.

The work discussed in chapter 2 aims to investigate the effect of phenological and morphological characteristics as EV on resource efficiency in wheat in the field and under controlled conditions, as well as to assess low cost phenotyping methodology in the field and under controlled conditions. Finally, genomic regions affecting EV should be identified to obtain a comprehensive overview of the potential impact of EV on water use efficiency. Since a in depth characterisation of agro-physiological traits is challenging under field conditions, due to strong dynamics in environmental factors, the application of various simulated drought stress conditions using precision phenotyping platforms, can be pivotal in the characterisation of ideally drought adapted genotypes. Chapter 3 focuses on the characterisation of key agro-physiological responses and water usage of sorghum under pre-anthesis drought conditions using a gravimetrical phenotyping platform. Additionally, the effects on plant structural growth and development, biomass and grain production were examined as well as the physiological mechanisms underlying drought adaptation in sorghum. As the primary organ for absorbing water and nutrients, crop root systems play a crucial role in the growth and development of plants. However, due to difficulties in the precise characterisation of below-ground traits, many breeding programs focus only on above-ground traits, which could possibly have led to an unintended decrease in root systems in European elite varieties. A suitable approach to overcome this problem, could be the introgression of genomic regions positively associated with root systems traits, using MAS. Chapter 4 presents the investigation of a QTL associated with an enhanced root system on nitrogen and water use efficiency in wheat under contrasting irrigation conditions. For this purpose, nitrogen partitioning was tracked using the ^{15}N tracer method via isotope discrimination in conjunction with a gravimetrical precision phenotyping platform.

2. Dissecting the Genetics of Early Vigour to Design Drought-Adapted Wheat

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Dissecting the Genetics of Early Vigour to Design Drought-Adapted Wheat

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Due to the climate change and an increased frequency of drought, it is of enormous importance to identify and to develop traits that result in adaptation and in improvement of crop yield stability in drought-prone regions with low rainfall. Early vigour, defined as the rapid development of leaf area in early developmental stages, is reported to contribute to stronger plant vitality, which, in turn, can enhance resilience to erratic drought periods. Furthermore, early vigour improves weed competitiveness and nutrient uptake. Here, two sets of a multi-reference nested association mapping (MR-NAM) population of bread wheat (*Triticum aestivum* ssp. *aestivum* L.) were used to investigate early vigour in a rain-fed field environment for 3 years, and additionally assessed under controlled conditions in a greenhouse experiment. The normalised difference vegetation index (NDVI) calculated from red/infrared light reflectance was used to quantify early vigour in the field, revealing a correlation ($p < 0.05$; $r = 0.39$) between the spectral measurement and the length of the second leaf. Under controlled environmental conditions, the measured projected leaf area, using a green-pixel counter, was also correlated to the leaf area of the second leaf ($p < 0.05$; $r = 0.38$), as well as to the recorded biomass ($p < 0.01$; $r = 0.71$). Subsequently, genetic determination of early vigour was tested by conducting a genome-wide association study (GWAS) for the proxy traits, revealing 42 markers associated with vegetation index and two markers associated with projected leaf area. There are several quantitative trait loci that are collocated with loci for plant developmental traits including plant height on chromosome 2D ($\log_{10}$ (P) = 3.19; PVE = 0.035), coleoptile length on chromosome 1B ($-\log_{10}$ (P) = 3.24; PVE = 0.112), as well as stay-green and vernalisation on chromosome 5A ($-\log_{10}$ (P) = 3.14; PVE = 0.115).

Keywords: *Triticum aestivum*, normalised difference vegetation index, NDVI, nested association mapping, genome-wide association study, GWAS

Abbreviations: Abbreviations; CoV, coefficient of variation; DAS, days after sowing; EV, early vigour; GH, greenhouse; GRAMMAR, genome-wide rapid association using mixed model and regression; GWAS, genome-wide association study; L1, leaf one; L2, leaf two; LMM, linear mixed model; MAF, minor allele frequency; Ma-NAM, Mace derived nested association mapping population; MR-NAM, multi-parent nested association mapping population; N, nitrogen; NDVI, normalised difference vegetation index; PC, principal component; PLA, projected leaf area (measured using the green pixel counter); Sc-NAM, scout derived nested association mapping population; SD, standard deviation; Su-NAM, Suntop derived nested association mapping population; TLA, total leaf area (= calculated area of L1 + L2); Var, variance.

INTRODUCTION

Global climate change is considered one of the biggest and most complex challenges the mankind has faced. One effect that has been observed since 1970, which leads to severe yield losses, is the increased occurrence of erratic drought phenomena (Liu et al., 2019). Specifically, the agricultural sector is facing serious challenges since drought-stress is considered the most limiting factor in rain-fed cropping systems (Hu and Xiong, 2014). Based on calculations of the Intergovernmental Panel on Climate Change (IPCC), it is predicted that the global mean surface temperature will rise 2°C more in the 20-year period from 2046 to 2065, than in the comparable period between 1986 and 2005, with a total increase of 4.8°C by 2100 (IPCC, 2015). As a result, more frequent heat and drought events are to be expected and to be classified as a major threat to the primary production sector in general and the wheat production in particular (Steinfort et al., 2017). Based on crop modelling scenarios, it is predicted that global wheat production will fall by 6% per 1°C temperature increase (Asseng et al., 2013). In particular, the Australian wheat production region is expected to experience a strong increase in drought and heat events, with a yield decrease of up to 20% projected from a median temperature increase of 2°C (Asseng et al., 2015). The severe impact of strong drought events has already been observed during the "Millennium Drought" between 2001 and 2009, where major reductions in production were recorded. In southern Australia in particular, production was severely decreased due to the impact of drought during this phase (Dijk et al., 2013). Large areas of the southern and western wheat-cropping regions in Australia have a Mediterranean climate, which is defined by terminal droughts (Siddique et al., 1990; Whan et al., 1991; Botwright et al., 2002; Rebetzke et al., 2008; Sadras and Dreccer, 2015; Rebetzke et al., 2017).

Consequently, there has been a growing focus on commercial and public breeding programs to identify traits associated with water-use efficiency to increase the yield potential under water-limited conditions (Lopes and Reynolds, 2012; Passioura, 2012). Potential traits of interest include long coleoptiles, which enable deeper sowing in the soil profile and improved access to water reservoirs underneath a dry surface soil, as well as reduced tillering to lessen unnecessarily metabolism into the non-fertile emerging tillers (Richards et al., 2010). Another is early vigour (EV), defined as the rapid production of leaf area during the early development phase of the plant (López-Castañeda et al., 1996). The primary advantage of EV is the increased biomass production early in the season and the rapid closure of the canopy, which can reduce evaporation of soil water, which then increases water availability (López-Castañeda and Richards, 1994; Condon et al., 2004). Early canopy closure also leads to minimized solar radiation on the soil and to an enhanced competitiveness of the crop against weeds (Dingkuhn et al., 1999; Coleman et al., 2001; Lemerle et al., 2001; Bertholsson, 2005). Mediterranean growing areas are water-limited environments which are characterized by experiencing late seasons droughts. According to Richards et al. (1987) and López-Castañeda et al. (1995), EV offers great potential for increasing water-use efficiency in such drought-prone regions. Additional advantages that may be associated with EV include larger uptake of essential plant nutrients, superior tolerance to aluminium stress, as well as improved yield under high temperatures and elevated atmospheric CO_2 concentration (Coleman et al., 2001; Lemerle et al., 2001; Liao et al., 2004; Bertholsson, 2005; Ludwig and Asseng, 2010; Valle and Calderini, 2010; Ryan et al., 2015). Previous studies in wheat have already identified various physiological traits associated with EV, such as the embryo size (Moore and Rebetzke, 2015), coleoptile length (Clarke et al., 1991; Rebetzke et al., 2007), tiller size, and leaf characteristics (Rebetzke and Richards, 1999; Rebetzke et al., 2007, 2017).

Several studies have highlighted the beneficial effect of increased EV on yield performance in specific environments. Nevertheless, due to insufficient knowledge about genetic variation and lack of information on the economic value of the trait, EV has only been introduced into breeding programs to a limited degree (Rebetzke et al., 2017). However, Botwright et al. (2002) demonstrated the positive effect of EV on yield performance in medium and low rainfall regions in combination with favourable soil conditions, such as sandy soils. Early vigour has significant implications for water demand. For example, a slight increase in leaf area growth during the vegetative growth stages can lead to an increase in biomass, transpiration area, and water use (Asseng and van Herwaarden, 2003). This can result in rapid depletion of soil water prior to anthesis, which may have a negative impact on grain yield during flowering time and grain filling (Richards and Townley-Smith, 1987). According to Turner and Nicolas (1998), strong vigorous genotypes have deeper and greater water uptake compared to less vigorous genotypes and are subsequently considered to be advantageous in low rainfall environments, including Mediterranean growing regions. However, despite a clear genetic effect on EV, its interaction with the environment, soil type, and available fertilizer is substantial. For example, EV may adversely affect yield if managed unfavourably. Therefore, ensuring that the crop is provided with sufficient nitrogen (N) is essential in order to prevent premature N deficiency due to excessive biomass production (Asseng and van Herwaarden, 2003).

To determine the impact of EV on yield, a better understanding of the physiological mechanisms of the trait along with its genetic control is required. In addition, efficient and low-cost phenotyping procedures are needed to assess EV in wheat. The aim of this study was (i) to investigate the physiological characteristics that drive EV in wheat in the field and under controlled conditions, (ii) to assess the efficiency of phenotyping methodologies under greenhouse and field conditions, and (iii) to identify genomic regions influencing EV in wheat.

MATERIALS AND METHODS

Plant Material

To evaluate EV in the field, a set of 685 spring wheat genotypes (further referred to as Set 1) was randomly selected from a multi-reference nested association mapping population (MR-NAM).

The MR-NAM population was developed based on 11 diverse founder lines which were crossed with the commercially used wheat varieties Suntop (AGT), Scout (LPB), and Mace (AGT), then consequently adapted to the environmental conditions of the western, northern, and southern cropping regions of Australia, respectively (Richard et al., 2015). The founder lines were selected according to key traits, such as drought adaptation and stay-green (e.g., Dharwar Dry, Drysdale), root architecture traits (e.g., Seri) or adaptation to nematodes, and disease resistance (e.g., Wylie, Gregory) (Richard et al., 2015; Christopher et al., 2021). After crossing the founder lines with the three parental lines, using an incomplete crossing scheme, the 15 F_1 lines were generated. Subsequently, these 15 F_1 lines were used for population development through inbreeding, which produces1474 F_4-derived lines and were then segmented into 15 genetically diverse families. These 15 families comprised four Mace-derived families, forming a conventional NAM population which was denoted as the Mace-NAM (Ma-NAM) component of the MR-NAM, five Scout-derived families (Sc-NAM), and six Suntop-derived families (Su-NAM). The NAM population was genotyped using the DArT-seq genotype-by-sequencing platform, producing over 25,000 polymorphic markers (Richard et al., 2015). The first set (SET 1) was tested in experimental years, 2015 and 2016, respectively. In 2017, a randomly selected subset comprising 210 lines (referred to as Set 2) was selected and was tested in the field in a greenhouse (GH) environment. In order to provide a more detailed information of the physiological characteristics, a core set was formed within Set 2, which was intensively investigated in the field and in the GH experiment.

Experimental Design

Evaluating Early Vigour Under Field Conditions

Field trials were conducted over 3 years from 2015 to 2017. All field trials were carried out under rain-fed conditions at the Hermitage Research Facility (HRF), Warwick, Queensland, Australia (28.21°S, 152.10°E, 480 m above sea level). The HRF site is characterized by alkaline, cracking, and heavy clay soils with high water-holding capacity. Cropping season is from May to October, with an average rainfall of 211 mm and an average temperature of 14°C. Further information regarding the environmental conditions is given in **Table 1**. Sites were sown with yield plots, each plot measuring 2 m × 6 m, containing 7 rows at 25 cm spacing, with a target crop density of 100 plants/m^2. To precisely reach the target crop density, thousand seed weight and germination rate were determined for seed of each genotype, while the sowing rates were calculated for each genotype. To avoid any artefacts in seed size, the seeds of the lines used in each trial were sourced from a common site and the year of the seed propagation. Seeds were generated from fully irrigated seed-increase rows sown at 0.5 m row spacing and fertilizer was applied to provide for the non-limiting conditions for both water and nutrients. Furthermore, diseases and weeds were controlled as necessary. This allowed for the full potential seed size of each genotype to be expressed during seed production. In all trials, no specific selection for seed size were performed. In each year the trials received 120 kg/ha^{-1} of urea prior to sowing, and 40 kg/ha^{-1} of Starter Z® (Incitec Pivot Fertilisers, Southbank, VIC, Australia; 10.5% N, 19.5% P, 2.2% S, 2.2% Zn) was applied at sowing. Plant protection measures were applied as necessary. All field trials were designed as a partially replicated (p-rep) block design with percentage of partial replication of 34, 29, and 62% in 2015, 2016, and 2017, respectively.

Across the field trials, the normalised difference vegetation index (NDVI) was used as a quantitative measure for EV. The NDVI measurements were collected using a hand-held NTech Greenseeker® model 505, manufactured by NTech Industries, Ukiah, CA, United States. By attaching the device to the body by using a harness, the measurement could be carried out constantly at a height of 1 m. The NDVI has been reported as a very useful index for studying the dynamics of canopy development and of senescence patterns of wheat (Lopes and Reynolds, 2012; Christopher et al., 2014). This vegetation index has also been previously used to evaluate EV in wheat (Li et al., 2014). In this study, NDVI measurements were collected at 29 days after sowing (DAS) for all field trials.

In 2017, detailed measures were captured to fully understand the physiological properties. A core set consisting of 30 genotypes was compiled from Set 2 and was intensively examined at the time of the NDVI measurement. The core set included all founder and reference lines from the MR-NAM population, along with selected good-performing lines from other unpublished experiments. From each of the 30 genotypes, ten plants per plot were randomly sampled for each genotype, while the leaf characteristics were recorded by measuring the length and width of the first (L1) and the second leaf (L2). Subsequently, the approximate leaf area for L1 and L2 was calculated by multiplying the measured length by the width. The sum of the calculated leaf areas of L1 and L2 was then expressed as the total leaf area (TLA).

Evaluating Early Vigour Under Controlled Conditions

The GH experiment was conducted using 210 genotypes from Set 2. The greenhouse chamber temperature was set on 22°C during daytime and 17°C during night time, while the lighting conditions were set to provide a 12-h photoperiod. Furthermore,

TABLE 1 | Details for environmental conditions for wheat trials subjected to analyses in this study.

Trial	Location	State	Sowing date	CIR [mm]	PAWC [mm]	AvgT [°C]	RAD (MJ m^{-2})
NAM 2015	HRF, Warwick	QLD	11.06.2015	103.4	329.6	13.1	2,318
NAM 2016	HRF, Warwick	QLD	22.07.2016	303.1	212.1	14.9	2,430
NAM 2017	HRF, Warwick	QLD	27.05.2017	112.4	286.47	14.2	2,276

Shon are trials name and year (Trial), location and state, sowing date, cumulative in-crop rainfall in mm (CIR), plant available water capacity of the soil in mm (PAWC), daily average temperature from sowing to maturity in °C (AvgT), and cumulative radiation from sowing to maturity in MJ m^{-2} (RAD).

the plants were irrigated on a daily basis. A single plant was grown per pot using 250 ml pots with 70 mm diameter. The potting media had a pH of 5.5–6.5 and was a composition of 70% pine bark (0–5 mm) and 30% coco peat, as well as fertilizers. Furthermore, Osmocote® (ICL SF, Sydney, NSW, Australia), containing 19.4% N, 16% P, and 5% K, was added to the potting media to guarantee sufficient nutrients during the experiment. The trial was designed as a fully replicated, randomized, and complete block design with six replications per genotype.

To measure leaf dimensions on a larger scale within the GH experiment setup, this study evaluated the extent of the image analysis methods that will be suitable for this purpose. For this purpose, a green pixel counter was programmed using the MATLAB® (MathWorks, Inc., Natick, MA, United States)

FIGURE 1 | Mode of operation of the green pixel counter, analysis takes place in two steps. First capture of the green pixels in the image as indicated by the red outlined area (left). Second, removal of all non-green pixels (right).

TABLE 2 | Descriptive statistics for collected field data for Set 1 (685 lines) and the subsets of lines Set 2 (210 lines) and core set (30 lines) captured at 21 and 29 days after sowing (DAS).

Set	Parameter	Unit	DAS	Year	Descriptive statistics				
					Mean	Min	Max	SD	CoV
Set 1	NDVI		29	2015	0.343	0.26	0.449	0.17	0.5
11	NDVI		29	2016	0.259	0.14	0.447	0.2	0.77
Set 2	NDVI		29	2015	0.341	0.26	0.456	0.03	0.09
12	NDVI		29	2016	0.339	0.27	0.461	0.04	0.11
13	NDVI		21	2017	0.325	0.22	0.394	0.04	0.12
14	NDVI		29	2017	0.264	0.19	0.416	0.03	0.13
Core Set	NDVI		29	2015	0.344	0.28	0.398	0.03	0.08
15	NDVI		29	2016	0.276	0.19	0.403	0.06	0.22
16	NDVI		21	2017	0.245	0.22	0.317	0.03	0.11
17	NDVI		29	2017	0.321	0.24	0.391	0.04	0.13
18	TLA	[cm^2]	21	2017	6.973	5.7	9.609	1.08	0.16
19	Leaf area L1	[cm^2]	21	2017	3.569	2.67	4.658	0.48	0.13
20	Leaf area L2	[cm^2]	21	2017	3.404	1.82	5.904	0.98	0.29
21	Length L1	[cm]	21	2017	10.83	8.59	13.12	1.11	0.1
22	Width L1	[cm]	21	2017	0.33	0.3	0.39	0.03	0.08
23	Length L2	[cm]	21	2017	10.68	8.15	16.96	1.88	0.18
24	Width L2	[cm]	21	2017	0.315	0.22	0.41	0.05	0.15
25	TLA	[cm^2]	29	2017	9.971	6.66	12.78	1.46	0.15
26	Leaf area L1	[cm^2]	29	2017	4.221	2.75	5.62	0.63	0.15
27	Leaf area L2	[cm^2]	29	2017	5.75	3	7.793	1.04	0.18
28	Length L1	[cm]	29	2017	12.04	9.01	13.85	1.04	0.09
29	Width L1	[cm]	29	2017	0.349	0.3	0.42	0.03	0.09
30	Length L2	[cm]	29	2017	15.49	10.3	18.33	1.6	0.1
	Width L2	[cm]	29	2017	0.369	0.29	0.47	0.04	0.12

Shown are the set of lines examined (Set), parameter examined, unit (NDVI is an index without units), time of measurement in days after sowing (DAS), year of experiment as well as for each set examined, mean value, minimum (Min), maximum (Max), variance (Var), standard deviation (SD), and coefficient of variation (CoV).

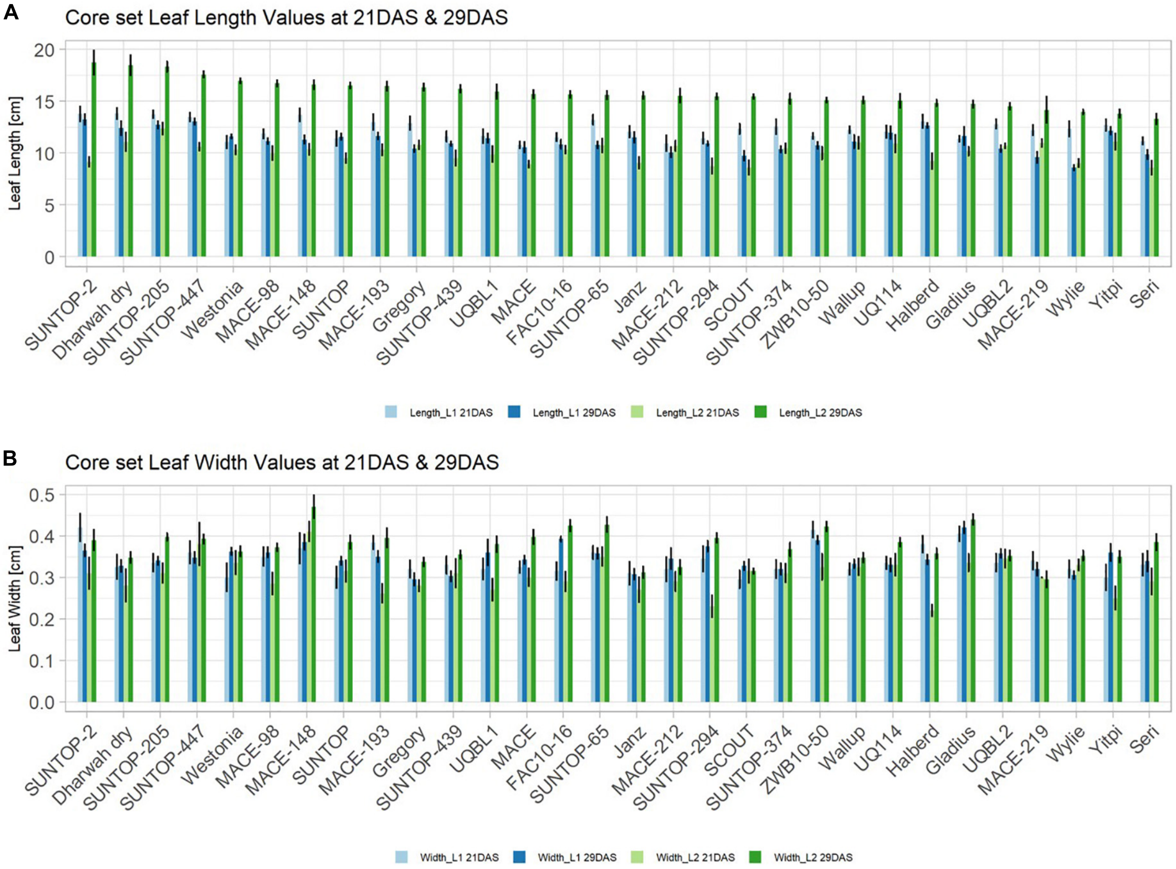

FIGURE 2 | Core set leaf parameters recorded under field conditions. **(A)** Showing leaf length values captured at 21 DAS and 29 DAS and **(B)** showing leaf width values captured at 21 DAS and 29 DAS. Error bars represent standard error.

programming language. The pixel counter identifies green pixels in an image to calculate a projected leaf area (PLA) and is a low-cost approach for image analysis (**Figure 1**). The measured PLA, using the green pixel counter, is analogous to the TLA, estimated as the sum of the areas of L1 and L2, as calculated from the length and width of each. With the default settings, images were taken using a Canon Eos 750D® camera. To reduce variation between images, the camera was mounted on a tripod at 80 cm distance from a platform on which every pot was placed. To avoid interference with other pixels or light sources, the image was taken in a closed room with consistent lighting conditions. In addition, a black background was placed behind the plant to exclude any other colour pigments from the picture, reducing them only to black and green pigments. The image analysis was conducted two times: first at 17 DAS and second at 21 DAS. At 17 DAS, images were captured for genotypes in Set 2. At 21 DAS all replications of the core set of Set 2 were imaged, as well as recorded manually. Again, the length and width of L1 and L2 were measured.

Statistical Analyses of Phenotype Data

For the calculation of the best linear unbiased estimators (BLUEs), the linear mixed model (LMM) described in Eq. 1 was used. For the calculation of the LMM, the R-language-based packages lme4[1] combined with lsmeans[2] were used.

$$P_{ijkl} = \mu + g_i + W_k + C_j + R_l + e_{ijkl} \quad (1)$$

where P_{ijkl} is the phenotypic value of the i^{th} genotype, in the k^{th} replication, μ stands for the overall mean, g_i describes the fixed effects of the i^{th} genotype. The random effects are W_k, which is the k^{th} replication, C_j, which represents the j^{th} column, and R_l representing the l^{th} row. The error term is represented by e_{ijkl}.

Pearson correlation matrices were created using the R package psych. Principal component analysis was conducted using the R package stats. For the Pearson correlation, as well as for the principal component analysis, we used the BLUEs described in Eq. 1.

Association Mapping

Association mapping was performed using the R package GenABEL (Aulchenko et al., 2007). Genome-wide rapid

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[1] http://CRAN.R-project.org/package=lme4

[2] https://cran.r-project.org/web/packages/lsmeans/index.html

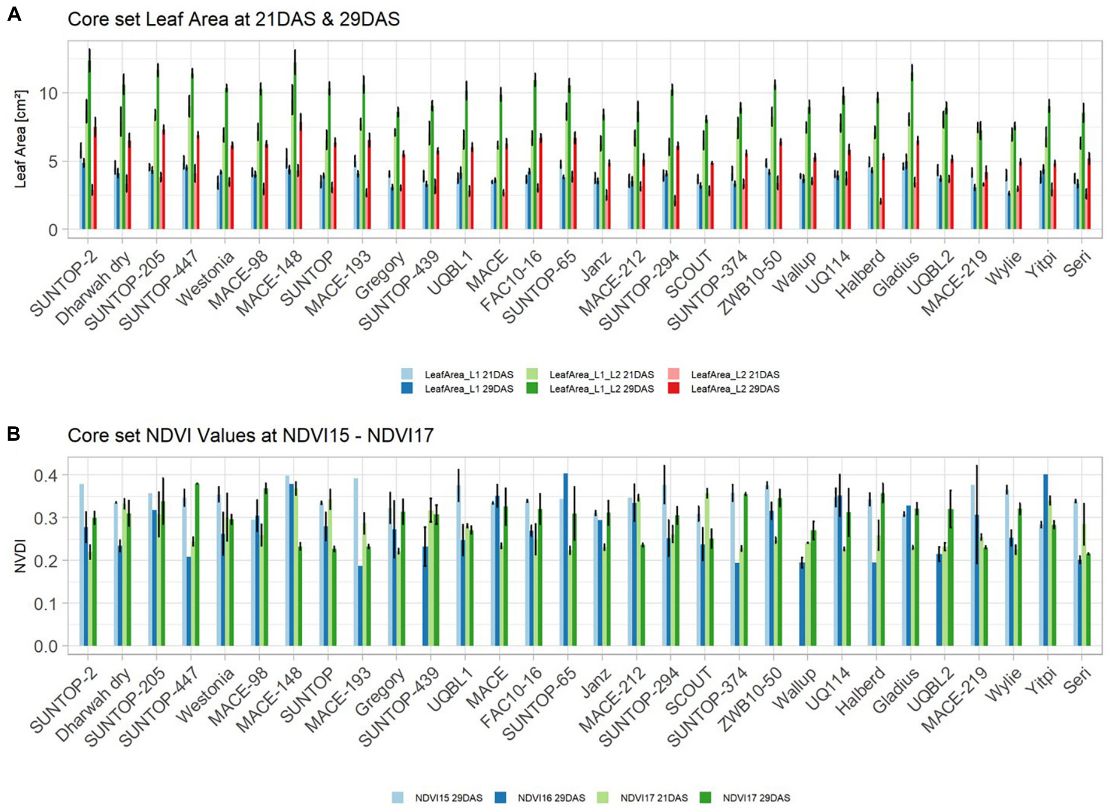

FIGURE 3 | (A) Core set leaf area parameters showing leaf area of leaf 1 (L1), leaf 2 (L2) as well as total leaf area (TLA) measured at 21 DAS and 29 DAS, respectively. **(B)** Core set NDVI parameters from NDVI15, NDVI16, and NDVI17 at 21DAS as well as NDVI17 at 29DAS. Error bars represent standard error.

association studies, using the mixed model and regression (GRAMMAR) method, initially estimate the residuals from the LMM on the assumption that the SNPs have no effect (null model). Subsequently, GRAMMAR then treats the residuals as phenotypes for further genome-wide analysis, using a standard linear mixed model (Zhou and Stephens, 2012). A total of 685 lines were genotyped using the presence/absence Diversity Arrays Technology genotyping-by-sequencing (SillicoDArTs^{TM}) platform. By applying zero mismatches and gaps, as well as a stringent alignment using BLASTN (Altschul et al., 1990), we were able to uniquely anchor 15,146 SNPs to a single position of the wheat Chinese Spring reference genome (RefSeq v1.0). The allelic association was calculated for NDVI, after accounting for population structure by implementing the first principal component, as well as the genome-wide kinship matrix for the genotypic trait values of NDVI and PLA. Prior to analysis, markers with more than 10% missing data or minor allele frequency (MAF) less than 5% were excluded from the analysis. A total number of 9,432 high-quality and polymorphic markers remained for the analysis. The cut-off value for markers being identified as significantly associated with the trait was set at the arbitrary threshold of $-\log_{10}(P) > 3$ which corresponds to a p cut-off of 0.001 significance level. The phenotypic variation explained by a given QTL (PVE) was calculated separately, according to Shim et al. (2015).

This study estimated broad sense (H^2) and narrow sense (h^2) heritability by using the R package sommer (Covarrubias-Pazaran, 2016). A marker-based approach to estimate σ^2_A, σ^2_D, by calculating additive and dominance relationship matrices, was applied. The models to estimate h^2 and H^2 are given in Eqs. 2, 3, respectively.

$$h^2 = \frac{\sigma^2_A}{\sigma^2_P} \tag{2}$$

$$H^2 = \frac{\sigma^2_A + \sigma^2_D}{\sigma^2_P} \tag{3}$$

With σ^2_A as the additive genetic variance, σ^2_D as the dominance genetic variance, and σ^2_P as the phenotypic variance.

RESULTS

Phenotypic Characterisation of Early Vigour

Expression of Early Vigour in the Field

Basic descriptive statistical indicators (minimum, maximum, and mean), variation (Var), standard deviation (SD), coefficient of variation (CoV) for NDVI, and leaf parameters of Set 1, Set 2, and the core set are all given in **Table 2**. For Set 1, the largest mean NDVI values were observed in 2015. For Set 2 and the core set, the largest mean values were reached in 2016, while in Set 2, the overall maximum mean NDVI was recorded in 2015. All leaf parameters which were measured in the core set showed larger values at 29 DAS compared to those recorded at 21 DAS (**Table 2**). Within the core set the largest values for L1 were reached by

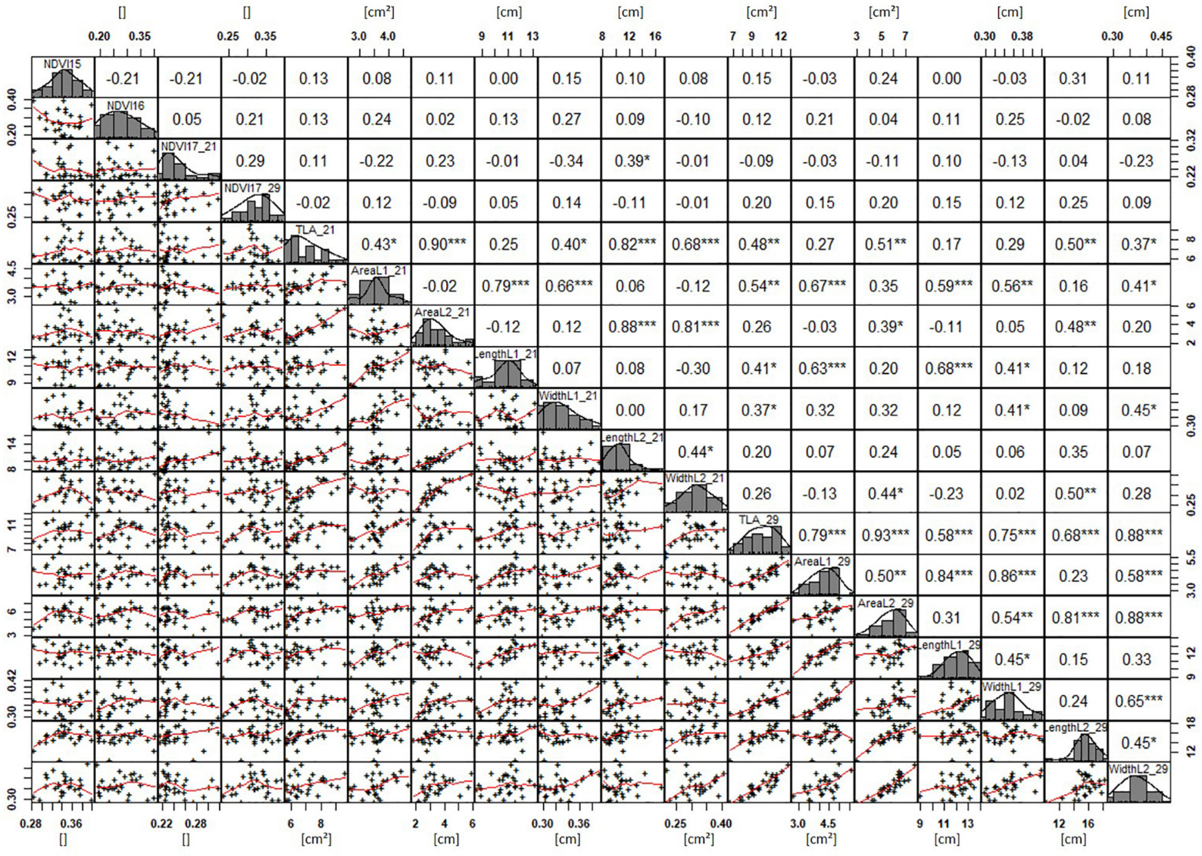

FIGURE 4 | NDVI and leaf parameters captured for the core set of 30 lines in field trials from 2015 to 2017. For each trait, the population distribution is displayed in the centre diagonal. The upper right shows the Pearson correlation coefficient for each trait combination. The lower left half shows the scatter plot with fitted line for each trait combination. * significant at $p < 0.05$; ** significant at $p < 0.005$; *** significant at $p < 0.001$.

TABLE 3 | Descriptive statistics for collected GH data for core set (30 lines) captured at 17 and 21 days after sowing (DAS).

Set	Parameter		DAS	Year	Descriptive statistics					
					Mean	**Min**	**Max**	**Var**	**SD**	**CoV**
Core Set	Seed weight	[g]	–	2017	0.095	0.068	0.121	0.000	0.012	0.129
31	PLA	[cm^2]	17 DAS	2017	13.895	8.236	19.681	7.740	2.913	0.210
32	PLA	[cm^2]	21 DAS	2017	14.795	10.635	20.348	4.350	2.158	0.146
33	Biomass	[g]	21 DAS	2017	0.067	0.010	0.108	0.001	0.024	0.364
34	TLA	[cm^2]	21 DAS	2017	11.254	6.915	16.493	5.659	2.518	0.224
35	Leaf area L1	[cm^2]	21 DAS	2017	3.707	1.983	5.125	0.714	0.904	0.244
36	Leaf area L2	[cm^2]	21 DAS	2017	7.548	4.600	12.203	2.933	1.799	0.238
37	Length L1	[cm]	21 DAS	2017	9.943	6.750	12.100	1.239	1.273	0.128
38	Width L1	[cm]	21 DAS	2017	0.366	0.267	0.483	0.003	0.060	0.164
39	Length L2	[cm]	21 DAS	2017	16.445	12.500	21.700	3.487	2.034	0.124
	Width L2	[cm]	21 DAS	2017	0.452	0.358	0.583	0.004	0.063	0.138

Shown are the set of lines examined (Set), parameter examined, unit, time of measurement (DAS), year of experiment as well as mean value, minimum (Min), maximum (Max), variance (Var), standard deviation (SD), and coefficient of variation (CoV).

Dharwah dry at 21 DAS and SUNTOP-2 at 29 DAS (**Figure 2A**). Furthermore, a significant ($p < 0.05$) difference between the genotypes was observed at 21 DAS and 29 DAS, with a smaller phenotypical variation at 21 DAS compared to the measurement at 29 DAS (**Supplementary Table 1**). For leaf length L2, similar results were observed with significant ($p < 0.05$) phenotypical variation at both time points, with a smaller variation at 21 DAS compared to 29 DAS (**Supplementary Table 1**). The largest leaf length L2 values were reached by SUNTOP-205 at 21 DAS and SUNTOP-2 at 29 DAS, respectively (**Figure 2B**). Regarding the leaf length of L1, the largest values were reached by SUNTOP-2 at 21 DAS and by Gladius at 29 DAS. For width L1, only at 29 DAS, a significant ($p < 0.05$) phenotypical variation could be observed (**Supplementary Table 1**). By comparing leaf area values calculated within the core set, it was observed that SUNTOP-2 showed the largest values for L1 at 21 DAS and 29 DAS, as well as for TLA at 21 DAS. For leaf area, L2 at 21 DAS, 29 DAS and TLA at 29 DAS, MACE-148 showed the largest values (**Figure 3**). For all leaf area traits which were recorded at 21 DAS, the phenotypical variation is smaller compared to the phenotypical variation at 29 DAS (**Supplementary Table 1**). In order to examine the impact of each leaf characteristic on the TLA, Pearson correlation coefficients for each factor were estimated (**Figure 4**). Person correlation analysis revealed a larger significant ($p < 0.001$) correlation between TLA at 21 DAS and leaf area L2 at 21 DAS ($r = 0.89$), than between TLA and leaf area L1 at 21 DAS ($p < 0.05$ $r = 0.42$). A similar correlation can be observed for L1 and L2 at 21 DAS, and L2 parameters at 21 DAS. Notably, a significant ($p < 0.05$) correlation was observed between NDVI 17 21 DAS and leaf length L2 at 21 DAS ($r = 0.39$). Furthermore, the relationship between the leaf area of L1 and L2 at 29 DAS and leaf parameters at 29 DAS shows similarities to the calculation made at 21 DAS. Leaf area L1 at 29 DAS shows stronger correlations with leaf TLA at 29 DAS ($r = 0.79$), compared to 21 DAS. However, the L2 area at 29 DAS ($r = 0.93$) shows a stronger correlation to TLA at 29 DAS. Interestingly, the correlation between area L1 and L2 at 21 DAS, and the L1 and L2 at 29 DAS ($r = 0.47$) is slightly smaller than the correlation with L2 area at 29 DAS ($r = 0.51$) and leaf length L2 at 29 DAS.

Expression of Early Vigour Under Controlled Conditions

In order to understand how EV can be determined under controlled conditions, this study conducted a trial testing Set 2 as well as the core set within a GH environment. Basic statistical indicators are given in **Table 3**. **Figure 5A** reveals that the leaf

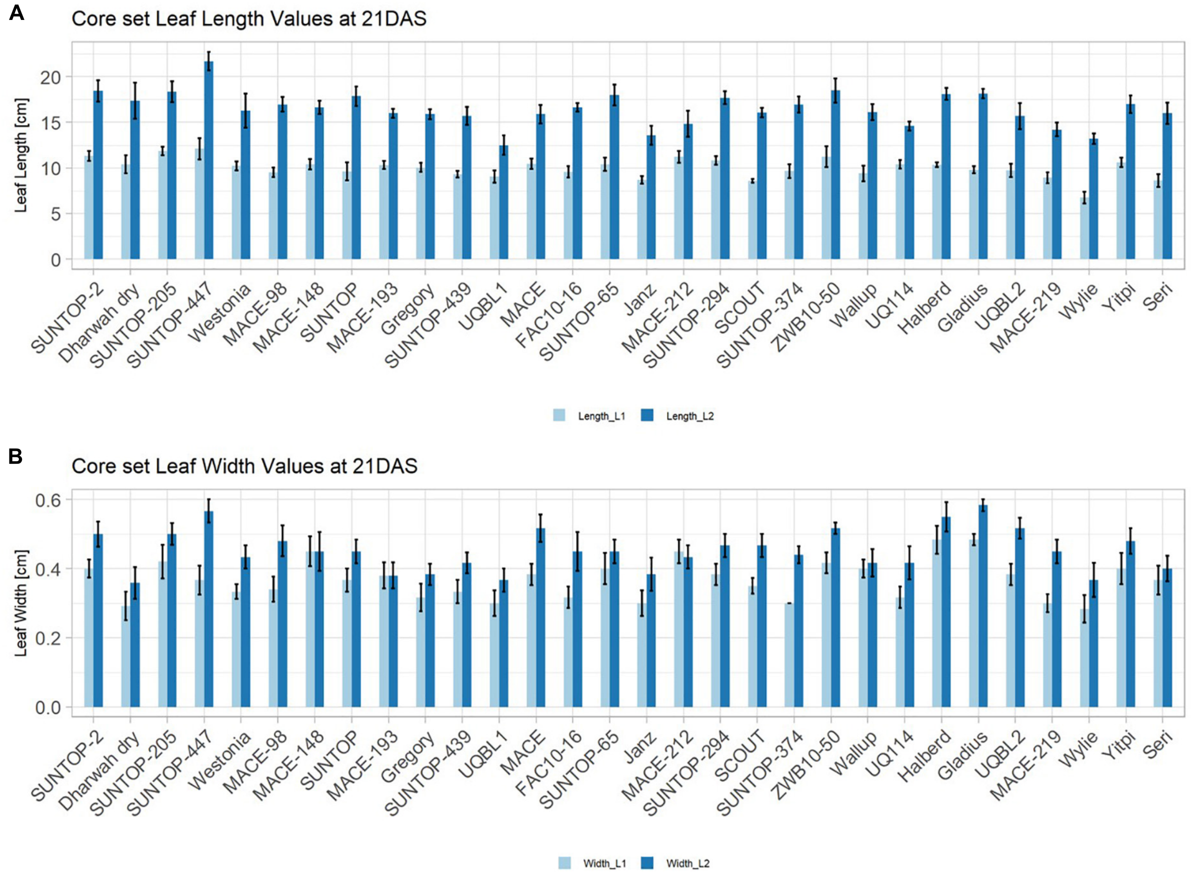

FIGURE 5 | Core set leaf parameters recorded under controlled conditions. **(A)** Showing leaf length values captured at 21 DAS and **(B)** showing leaf width values captured at 21 DAS. Error bars represent standard error.

length and leaf width of L2 exceeds L1 for almost all genotypes at 21 DAS. The only exceptions are the leaf widths of MACE-148 and of MACE-193, where L1 and L2 had similar values (**Figure 5B**). The largest leaf length values for L1 and L2 were reached by SUNTOP-447 (**Figure 5A**). As in field conditions, Gladius reached the largest values for leaf width L1 as well as for leaf width L2 (**Figure 5B**). Furthermore, for all the measured leaf parameters, significant ($p < 0.05$) differences can be observed. However, the recorded phenotypical differences show a lower variation compared to the field conditions (**Supplementary Table 2**). The calculated leaf area showed a significant ($p < 0.05$) phenotypical difference, particularly the L2, as well as the L1&L2 values (**Supplementary Table 2**). The SUNTOP-447 exhibited the largest values for leaf length, leaf area L2, and L1&L2, respectively. Maximum leaf area and L1 were exhibited by MACE-212 (**Figure 6A**). Only around two-thirds of the core set showed increased PLA at 21, compared to 17 DAS (**Figure 6B**). Pearson coefficients of correlation showed a significant positive relationship of the collected dry matter with the PLA 17 DAS ($p < 0.05$; $r = 0.46$) and with PLA 21 DAS ($p < 0.001$; $r = 0.71$). Furthermore, the PLA at 21 DAS shows a positive correlation to leaf width L2 ($r = 0.34$), as well as to area L2 ($r = 0.38$) and TLA ($r = 0.39$). As under field conditions, the TLA of L1 and L2 is more affected by area L2 ($r = 0.96$). However, area L1 ($r = 0.85$) seems to have a greater impact on TLA in the GH compared to the field. Furthermore, it is noteworthy that dry mass measured at harvest at 21 DAS shows a positive correlation to all captured leaf characteristics. Moreover, seed weight (SW) showed no significant correlation to any other trait (**Figure 7**).

Components of Early Vigour

To determine which physiological parameters of the leaf had the greatest influence on EV, a principal component (PC) analysis was performed. The analysis was conducted for the core set data and included parameters such as the leaf measurements at 21 DAS and at 29 DAS in the field, or at 21 DAS in the GH, as well as dry matter content, grain weight, and the respective NDVI values from 2015 to 2017 (**Figure 8**). For the field data, the NDVI in 2015 (NDVI 15) was largely associated with parameters connected to L2 at 21 DAS, and to leaf length and area of L2 at 29 DAS. The NDVI 16 and NDVI 17 at 29 DAS showed a strong association with area L1 at 21 DAS and area L1 at 29 DAS. In this regard, it became apparent that several parameters recorded at 29 DAS, such as area L1 and L2 and leaf width L2, were strongly correlated as well. Interestingly, for NDVI at 21 DAS, no association to any physiological parameters of the leaf was observed. In the GH, it could be observed that area L1 and L2 were slightly and closely more associated with area L2 than with area L1. Furthermore,

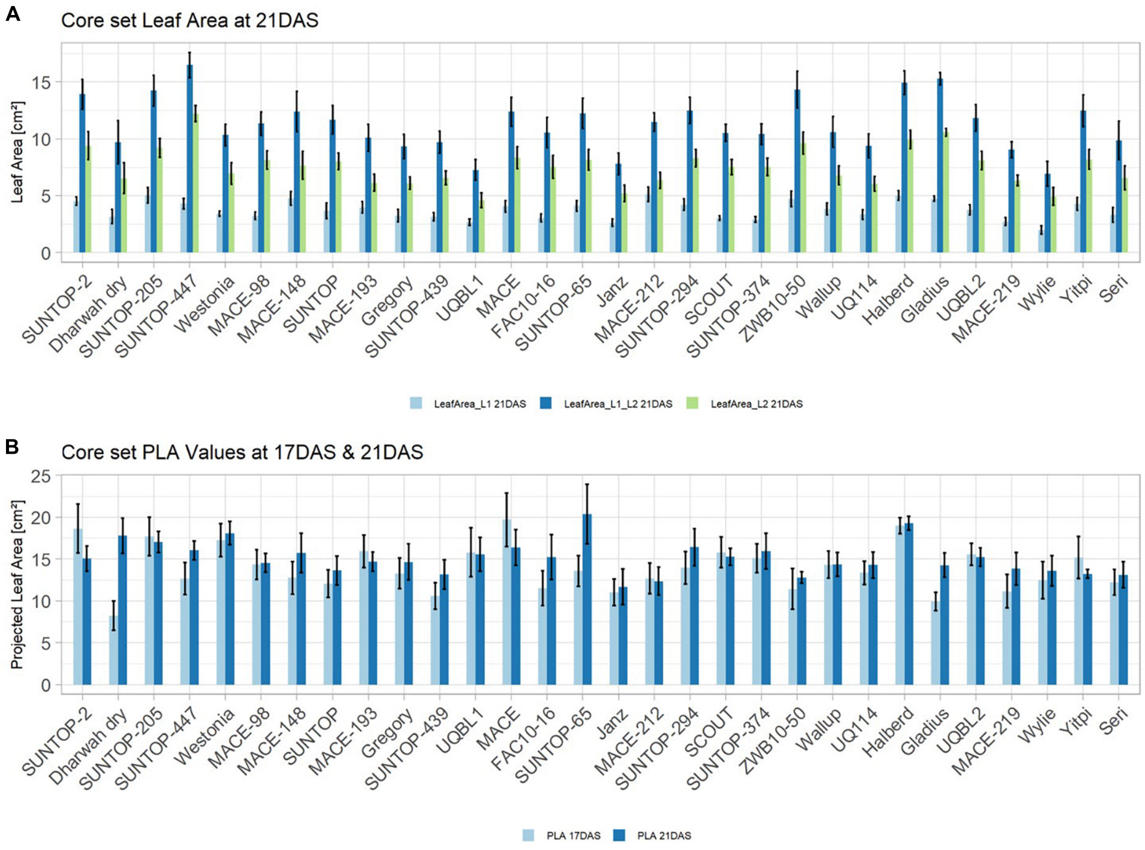

FIGURE 6 | (A) Core set leaf area parameters showing leaf area of leaf 1 (L1), leaf 2 (L2) as well as total leaf area (TLA) measured at 21 DAS in greenhouse experiment in 2017. **(B)** Core set projected leaf parameters (PLA) recorded at 17DAS and 21DAS. Error bars represent standard error.

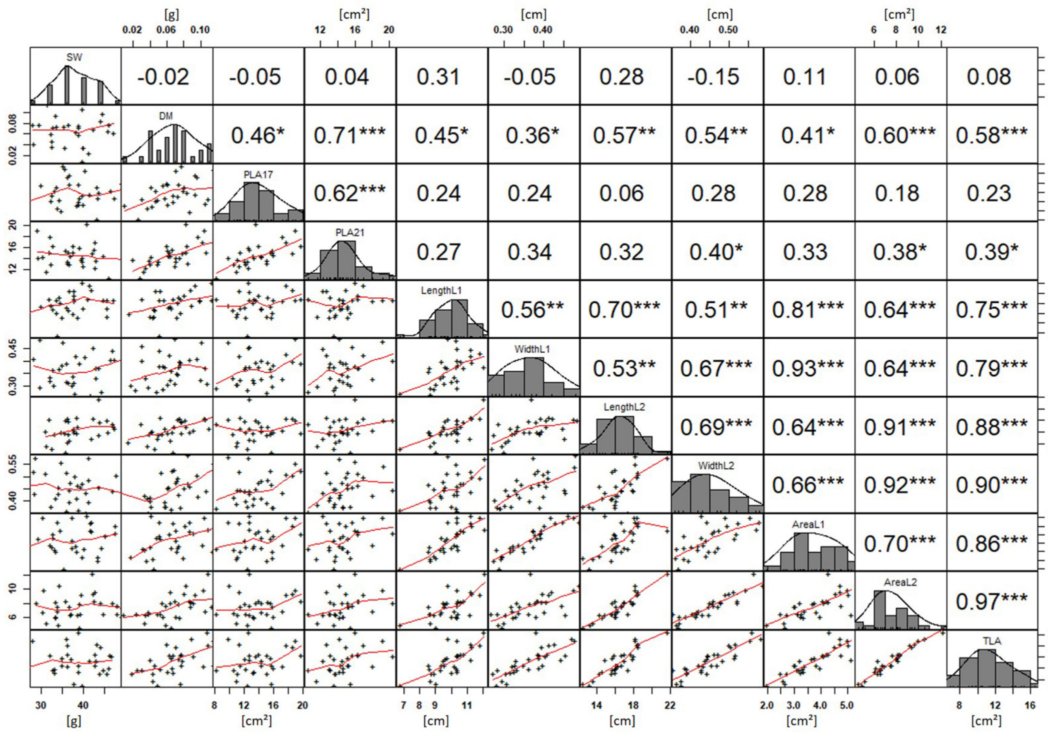

FIGURE 7 | Projected leaf area (PLA) by green pixel count method and leaf parameters captured for core set in greenhouse experiment. For each trait, the population distribution is displayed in the centre diagonal. The upper right shows the Pearson correlation coefficient for each trait combination. The lower left half shows the scatter plot with fitted line for each trait combination. * significant at $p < 0.05$; ** significant at $p < 0.005$; *** significant at $p < 0.001$.

area L2 appeared to be in a stronger association to leaf width L2 than to leaf length L2. The PLA at 17 DAS and PLA at 29 DAS showed a strong association to each other. However, apart from dry mass, no significant associations to any of the leaf parameters were observed.

Identifying Genetic Determinants of Early Vigour

Genetic analysis was performed with NDVI values for the 685 genotypes from Set 1 in 2015 (NDVI'15) and in 2016 (NDVI'16), along with NDVI values from 2015 to 2017, and the PLA data from the GH trial in 2017 in the 221 genotypes of Set 2. Several SNP markers exceeded the arbitrary threshold of association ($-\log_{10}(P) = 3$) for NDVI and PLA across all years in the genome-wide association study. A total of 41 QTL were associated [$-\log_{10}(P) = 3$] with either NDVI or PLA. The majority of trait-associated SNP markers (21) were associated with NDVI at 21 DAS in 2017 in Set 2 (**Figure 9**). Chromosome positions of all identified marker-trait associations for NDVI and PLA in the different environments are summarized in **Table 4**. All the identified QTL PVE was low and was ranged between 0.024 to 1.35%. The five most significant QTL, *QSG.qwr-3B.1*, *QSG.qwr-2A.3*, *QSG.qwr-3D.1*, *QSG.qwr-1A.1*, and *QSG.qwr-5B.2* accounted for 0.4% of the variation (**Table 4**). Most QTL effects were small and few, as QTL were detected in multiple environments or across traits, reflecting the genetic complexity and the strong environmental dependency of EV traits. This finding is consistent with several other studies that have also identified multiple QTL for several EV related traits located on different chromosomes (**Table 5**). In accordance with this observation, the narrow-sense heritability of both NDVI in Set 1 was found to be moderate with $h^2 = 0.22–0.28$, and small for NDVI and PLA in Set 2 with $h^2 = 0.08–0.04$ (**Table 6**).

DISCUSSION

The first objective of this study was to provide information on physiological components that contribute to EV of wheat, both in the field and under controlled conditions. Several studies observed a strong contribution of leaf width to a specific leaf area, and consequently to EV (Rebetzke and Richards, 1999; Richards and Lukacs, 2002; Maydup et al., 2012). Our results showed that the variation in EV is strongly associated with the leaf length of both L1 and L2 in the field, as well as in the GH. For both leaves (L1 and L2), leaf length is the main contributor to a larger leaf area development, and, consequently, to an increased EV. Furthermore, leaf length of L2 showed a slightly greater impact on the area of L1 and L2 with an advancing development. This finding agrees with other studies reporting a significant positive correlation between the area of L2 and the leaf length (Nursinow et al., 2011; Boden et al., 2014; Moore and Rebetzke, 2015; Duan et al., 2016). The data revealed faster development of L2 in the

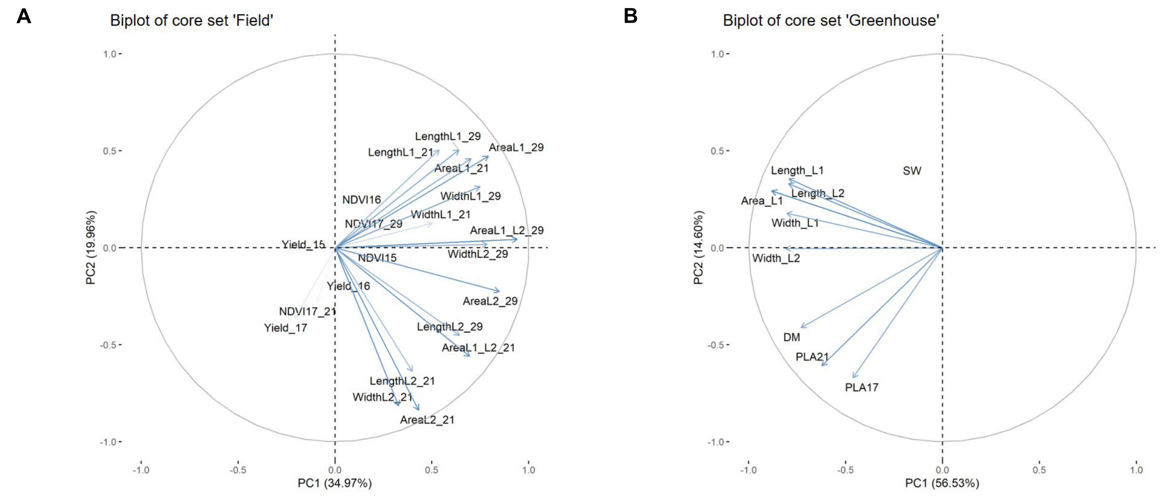

FIGURE 8 | Biplot from principal component analysis for core set field **(A)**, core set greenhouse **(B)**.

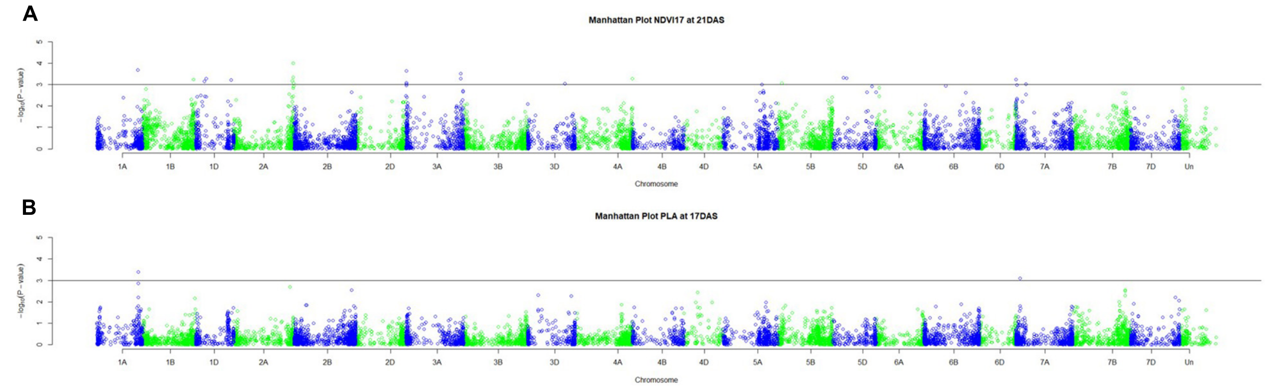

FIGURE 9 | Manhattan plots for **(A)** NDVI17 at 21DAS and **(B)** PLA at 17DAS. The horizontal line at $-\log_{10}(P) = 3$ indicates the chosen probability threshold to indicate a significant association with the trait.

GH compared to the field studies. This was also observed by Rebetzke et al. (2007) and leads to the conclusion that EV can be recorded at earlier stages under controlled conditions than in the field. Previous studies have reported that embryo size is a highly heritable trait that is strongly associated with leaf area (López-Castañeda et al., 1996; Rebetzke and Richards, 1999; Aparicio et al., 2002; Moore and Rebetzke, 2015). Since it has been established that embryo size increases with seed weight in wheat (Moore and Rebetzke, 2015) and barley (López-Castañeda et al., 1996), this study used seed weight to indirectly evaluate the impact of embryo size on EV. In the GH experiment, it was not possible to demonstrate the positive impact of embryo size on EV by using seed weight, since no significant correlation with any measured leaf parameter was observed. Nonetheless, several studies reported a positive effect of the embryo size on early vigour in wheat (Rebetzke and Richards, 1999; Richards and Lukacs, 2002; Moore and Rebetzke, 2015). In other studies, however, the total variation in EV could not be exclusively explained by the considering seed weight (Maydup et al., 2012), and the seed density has also been suggested as a potentially more decisive factor in determining EV (Ball et al., 2011). This partially explains why no correlation between seed weight and EV parameters could be found in the present study. In the GH experiment, biomass was recorded and was exhibited as a significant correlation with the area of L2, as well as area of L1 and L2, suggesting an increased above-ground biomass for genotypes with greater EV. Richard et al. (2019) reported that lines with increased above-ground biomass were strongly associated with an increased grain yield.

The second objective of this study was to evaluate high-throughput methods to precisely record physiological leaf characteristics in field and greenhouse trials. Mullan and Reynolds (2010) reported a significant correlation between NDVI, leaf area, and biomass. These PCA results from the current study tended to confirm that NDVI and the leaf area are related. Although the biomass in the field was not separately measured, a significant positive correlation between biomass and leaf area in the GH experiment suggested a putative relationship. In the

TABLE 4 | QTL identified with significant association to phenotypic traits (*P*-value > −log10 (*P*) = 3).

QTL name	Chr	Position [bp]	Trait	DAS	Year	*P*-value	PVE	No. of reported genes in ± 20kb	Gene-ID	Start position [bp]	End position [bp]
QSG.qwr-1A.1	1A	530870345	NDVI	21	2017	3.68	0.135	1	TraesCS1A02G341200	530883540	530884786
QSG.qwr-1A.2	1A	537895686	PLA	17	2017	3.39	0.125	–	NA	NA	NA
QSG.qwr-2A.1	2A	758570584	NDVI	21	2017	3.17	0.114	3	TraesCS2A02G552800	758553979	758559790
									TraesCS2A02G552900	758583247	758587695
									TraesCS2A02G553000	758588329	758589199
QSG.qwr-2A.2	2A	768989573	NDVI	21	2017	3.34	0.120	3	TraesCS2A02G573200	768981429	768983083
									TraesCS2A02G573300	768996829	768998744
									TraesCS2A02G573400	769002934	769004935
QSG.qwr-2A.3	2A	769344238	NDVI	21	2017	4	0.152	1	TraesCS2A02G574300	769340976	769344226
QSG.qwr-2A.4	2A	775175704	NDVI	21	2017	3.06	0.113	1	TraesCS2A02G583000	775165226	775166200
QSG.qwr-3A.1	3A	8267133	NDVI	21	2017	3.08	0.107	2	TraesCS3A02G008800	8253788	8255463
									TraesCS3A02G008900	8258758	8260510
QSG.qwr-3A.2	3A	9212918	NDVI	21	2017	3.64	0.134	1	TraesCS3A02G011400	9210008	9210976
QSG.qwr-3A.3	3A	9463808	NDVI	21	2017	3.03	0.107	3	TraesCS3A02G011800	9444895	9445740
									TraesCS3A02G011900	9473058	9473828
									TraesCS3A02G012000	9480858	9481594
QSG.qwr-3A.4	3A	711365648	NDVI	21	2017	3.5	0.122	1	TraesCS3A02G480500	711380680	711385803
	3A	711366350	NDVI	21	2017	3.27		–	NA	NA	NA
QSG.qwr-4A.1	4A	744471786	NDVI	21	2017	3.28	0.119	1	TraesCS4A02G499600	744456472	744458084
QSG.qwr-5A.1	5A	526386152	NDVI	29	2015	3.14	0.115	5	ENSRNA050011196	526378014	526378087
									ENSRNA050021751	526384607	526384709
									TraesCS5A02G315700	526393365	526396850
									TraesCS5A02G315800	526396904	526400499
									TraesCS5A02G315900	526400750	526402134
QSG.qwr-5A.2	5A	529810097	NDVI	29	2015	3.54	0.025	–	NA	NA	NA
QSG.qwr-5A.3	5A	592515889	NDVI	29	2015	3.07	0.030	1	TraesCS5A02G398700	592518737	592520426
QSG.qwr-6A.1	6A	5480626	NDVI	29	2015	3.14		1	TraesCS6A02G011800	5473439	5480862
QSG.qwr-7A.1	7A	263934	NDVI	21	2017	3.24	0.112	2	TraesCS7A02G000200	242359	246258
									TraesCS7A02G000300	250565	253987
QSG.qwr-7A.2	7A	51498553	PLA	17	2017	3.1	0.112	–	NA	NA	NA
QSG.qwr-7A.3	7A	130596094	NDVI	21	2017	3.03	0.106	1	TraesCS7A02G177400	130608975	130613414
QSG.qwr-7A.4	7A	694049656	NDVI	29	2016	3.63	0.040	2	TraesCS7A02G506800	694053373	694053943
									TraesCS7A02G506900	694060394	694067693
QSG.qwr-1B.1	1B	37762680	NDVI	29	2016	3.12	0.037	–	NA	NA	NA
QSG.qwr-1B.2	1B	84949441	NDVI	29	2016	3.47	0.039	–	NA	NA	NA
QSG.qwr-1B.3	1B	658902240	NDVI	21	2017	3.24	0.113	3	TraesCS1B02G434300	658908133	658910735
									TraesCS1B02G434400	658911233	658914898
									TraesCS1B02G434500	658915051	658919932
QSG.qwr-3B.1	3B	22137315	NDVI	29	2016	4.24	0.051	1	TraesCS3B02G042800	22129256	22130588
QSG.qwr-5B.1	5B	52306024	NDVI	21	2017	3.06	0.114	1	TraesCS5B02G046800	52317367	52318139
QSG.qwr-5B.2	5B	162778081	NDVI	29	2015	3.64	0.030	1	TraesCS5B02G110800	162771499	162776936
QSG.qwr-5B.3	5B	576298790	NDVI	29	2015	3	0.024	–	NA	NA	NA
QSG.qwr-7B.1	7B	748033995	NDVI	29	2015	3.27	0.026	1	TraesCS7B02G497400	748012557	748015135
QSG.qwr-1D.1	1D	52680610	NDVI	29	2016	3.2	0.035	1	TraesCS1D02G072300	52658816	52664518
QSG.qwr-1D.2	1D	111980607	NDVI	21	2017	3.15	0.112	1	TraesCS1D02G116200	111967743	111980590
QSG.qwr-1D.3	1D	134460288	NDVI	21	2017	3.28	0.120	–	NA	NA	NA
QSG.qwr-1D.4	1D	461051177	NDVI	21	2017	3.21	0.112	1	TraesCS1D02G389300	461052237	461057083
QSG.qwr-2D.1	2D	19551003	NDVI	29	2016	3.19	0.035	3	TraesCS2D02G051300	19543541	19544843
									TraesCS2D02G051400	19555735	19557093
									TraesCS2D02G051500	19561151	19563368
QSG.qwr-2D.2	2D	69503900	NDVI	29	2017	3.05	0.100	2	TraesCS2D02G120100	69502005	69504329
									TraesCS2D02G120200	69504557	69510365
QSG.qwr-2D.3	2D	362486328	NDVI	29	2017	3.11	0.108	–	NA	NA	NA
QSG.qwr-3D.1	3D	325183855	NDVI	29	2015	3.76	0.032	–	NA	NA	NA
QSG.qwr-3D.2	3D	481926113	NDVI	21	2017	3.04	0.113	1	TraesCS3D02G368700	481908948	481926630
QSG.qwr-5D.1	5D	138341788	NDVI	21	2017	3.31	0.115	–	NA	NA	NA
QSG.qwr-5D.2	5D	179786153	NDVI	21	2017	3.3	0.115	1	TraesCS5D02G123000	179762666	179778583
QSG.qwr-6D.1	6D	50627689	NDVI	29	2015	3.35	0.027	–	NA	NA	NA
QSG.qwr-7D.1	7D	533491186	NDVI	29	2015	3.11	0.026	–	NA	NA	NA

Shown are QTL identifier, chromosome location (Chr), Positions refer to physical positions [bp] on reference genome Chinese Spring (RefSeq v1.0), trait, time of measurement (DAS), year of measurement (Year), probability of association with trait by chance [−log10 (p-value)], phenotypic variation explained by a given QTL (PVE), number of genes with 20 kb of the QTL SNP location (No. of genes ± 20 kb), name of genes reported within the 20 kb radius of the QTL SNP (Gene-ID), start and end position of reported gene in [bp] (Start Position [bp]/ End Position [bp]).

TABLE 5 | Summary of early vigour related QTL reported in previous publications.

Trait	QTL/marker name	Chr	Publication
Coleoptile length	QClp.ipk-1A	1A	Landjeva et al., 2008
Coleoptile length	QClp.ipk-1B	1B	
Coleoptile length	ksuG9c	1A	Rebetzke et al., 2007
Coleoptile length	Stm55ltgag	2D	
Coleoptile length	psr426	5A	
Coleoptile length	psr326b	5D	
Embryo size	gwm18	1B	Moore and Rebetzke, 2015
EV, canopy temperature	41	3B	Bennet et al., 2012
Ground cover	QGCw.caas-1A.1	1A	Li et al., 2014
Ground cover	QGCw-caas-1D	1D	
Ground cover	QGCs-caas-2A.2	2A	
Ground cover	QGCs.caas-3B.1	3B	
Ground cover	QGCw-caas-5B	5B	
Ground cover	QGCw.caas-5B	5B	
Ground cover	QGCwcaas-5D	5D	
Ground cover	QGCscaas-6A	6A	
Ground cover	QGCs-caas-6A	6A	
Leaf length	gwm261	2D	Moore and Rebetzke, 2015
Leaf length	cdo669b	4B	
Leaf length	E36/M60-210-P1	2D	Steege et al., 2005
Leaf length	E48/M48-217-P2	5D	
Leaf length	E48/M60-225-P1	6D	
Leaf length	E45/M52-150-P1	7D	
Leaf width	wmc190	2D	Moore and Rebetzke, 2015
Leaf width	wmc289	5B	
Leaf width	E45/M52-274-P1	1D	Steege et al., 2005
Leaf width	Xgwm458	1D	
Leaf width	E42/M51-482-P2	2D	
Leaf width	Xgwm165	4D	
Leaf width	E42/M52-241-P1	5D	
Leaf width	E51/M52-189-P1	7D	
NDVI	QNDVIs-caas-3A	3A	Li et al., 2014
NDVI	QNDVIw-caas-6D	6D	
NDVI	QYld.aww-1B.2	1B	Tura et al., 2020
NDVI	QTgw.aww-1B	1B	
Relative growth rate	QRgr.saas-5A	5A	Li et al., 2017
Root dry weight	QRdw.saas-5A	5A	
Root length	QRlp.ipk-1A	1A	Landjeva et al., 2008
Root length	QRlp.ipk-7D	7D	
Shoot biomass	Rht-B1	4B	Ryan et al., 2015
Shoot biomass	Rht-D1	4D	
Shoot biomass	wmc525	7A	
Shoot fresh weight	QSfw.saas-5A	5A	Li et al., 2017
Shoot dry weight	QSdw.saas-5A	5A	
Total leaf area	QTla.saas-5A	5A	

Reported trait (Trait), name of the respective QTL or marker that is associated with the trait (QTL/Marker Name), chromosome on which the QTL or marker is located (Chr), cited publication which reported QTL (Publication).

TABLE 6 | Narrow-sense (h2) and broad-sense heritability for NDVI and PLA recorded in set 1 (685 lines) and set 2 (210 lines).

Set	Trait	DAS	Year	Heritability	
				h^2	H^2
Set 1	NDVI	29	2015	0.22	0.28
	NDVI	29	2016	0.28	0.38
Set 2	NDVI	29	2015	0.05	0.06
	NDVI	29	2016	0.04	0.05
	NDVI	21	2017	0.07	0.08
	NDVI	29	2017	0.06	0.07
	PLA	17	2017	0.04	0.04

Trait, time of measurement (DAS), year of measurement (Year), and narrow sense heritability (h^2) as well as broad sense heritability (H^2).

correlation analysis, only one significant correlation between NDVI and leaf parameters could be established, which was NDVI17 at 21 DAS and leaf length L2 at 21 DAS. This confirms that leaf length L2 contributes more to an increased EV compared to leaf dimensions of L1. This is also supported by other studies (López-Castañeda et al., 1996; Richards and Lukacs, 2002; Duan et al., 2016). In terms of high-throughput phenotyping methods for EV in GH environments, this study tested the ability of a green pixel counter as a low-cost method. Since embryo size strongly affects EV, the method is not able to explain the trait completely. However, the results for PLA calculated by the green pixel counter indicate great potential to measure EV under controlled conditions. In particular, the leaf parameters of L2 showed a significant correlation with PLA, as well as with the biomass. Furthermore, the green pixel counter was successfully used to measure coleoptile tiller length, a trait which strongly affects EV and is also highly correlated to embryo size.

The third aim of this study was to achieve a better understanding of EV genetics in wheat. The GWAS for Set 1 and Set 2 revealed 41 SNP markers for NDVI and for PLA, which were linked to 60 protein-coding regions across 17 chromosomes. Consistent with previous studies, the present study shows that EV in wheat is a quantitative trait with numerous QTL located across several chromosomes (Li et al., 2014; Moore and Rebetzke, 2015; Boudiar et al., 2016). Numerous studies have reported the effect of dwarfing genes on coleoptile length (Rebetzke et al., 2007; Li et al., 2017), coleoptile width (Rebetzke et al., 2014), and leaf epidermal cell dimension (Botwright et al., 2005). In most of these studies, the influence of dwarfing genes was highlighted. The gibberellic acid (GA)-insensitive dwarfing genes *Rht B1b on* chromosome 4B and *Rht D1db* on chromosome 4D have been reported to reduce coleoptile length and, consequently, EV, since they decrease epidermal cell length in leaf tissue (Ellis et al., 2004; Rebetzke et al., 2007; Yu and Bai, 2010; Li et al., 2017; Rebetzke et al., 2017). In our study, no SNP markers were detected on either one of the 4B or 4D chromosomes. This suggests that NDVI and PLA are the less affected traits by the presence of *Rht B1b* and of *Rht D1b*. Comparable results were reported in Li et al. (2014), were none of the parental lines that carried *Rht-B1b*, and only one parental line contained *Rht-D1b*, while no QTL was identified on chromosome 4D. However, a significant correlation could be observed between *Rht-D1* and NDVI and the ground cover in certain environments. The *Rht 8*, on the short arm of chromosome 2D, is a GA-responsive dwarfing gene reported to have a secondary effect of reducing epidermal cell length. Hence, it is more appropriate for achieving good canopy cover in combination with a semi-dwarf growth habit (Botwright et al., 2005). Chai et al. (2019) reported the WRKY transcription factor *TraesCS2D01G051500* as a possible candidate gene for *Rht8*. We identified QTL *QSG.qwr-2D.1* in the vicinity of *TraesCS2D01G051500*, and found this QTL to be significantly associated with NDVI17 at 29 DAS. Several studies have identified QTL associated with coleoptile length on chromosome 1B, including markers *XpGTG-mTCGA294* (Yu and Bai, 2010) and *wsnp_CAP11_c2596_1325540* (Ma et al., 2020). This study confirms that chromosome 1B is a region of interest for EV, since three significant QTL were detected on this chromosome. That applies particularly to *QSG.qwr-1B*, which is located at the same region on the long arm region of chromosome 1B as *wsnp_CAP11_c2596_1325540*, as reported in Ma et al. (2020). Another region of interest is chromosome 5A, which is also considered as the most important chromosome for stay-green traits (Shi et al., 2017; Liu et al., 2019), including the isopentenyl transferase gene (Gan and Amasino, 1995). Furthermore, chromosome 5A harbours several major developmental genes, such as the vernalisation gene *Vrn1*, frost resistance gene *Fr1,* as well as genes for ear emergence time and for the plant height (Sutka and Snape, 1989; Kato et al., 1999; Galiba et al., 1995).

The results of the current phenotypic investigation extended our insights into the EV trait in wheat. The key characteristics of EV and the relationship with other traits, such as biomass, were successfully identified. In addition, this study presents effective methods that can be used to detect EV in the field, as well as under controlled conditions. In particular, the connection between the leaf length parameters and the NDVI highlights the great potential of NDVI, especially if given recent advances in unmanned aerial vehicle or drone phenotyping platforms (Shi et al., 2016). Nevertheless, potential interactions due to environmental factors must be clarified by practical crop management for a better understanding, since factors such as sowing time, sowing depth, sowing rate, and row spacing may also influence EV. In particular, the interaction and value of EV in specific target environments must be clarified. Furthermore, it has to be considered that yield predictions based on EV can be very challenging. Since the trait is recorded at a very early developmental stage, its relationship to yield performance can subsequently be influenced by a multitude of complex environmental factors. For example, abiotic stress factors have a particularly decisive influence on yield and its yield components, especially during key developmental stages, such as tillering and flowering. Nevertheless, EV is essential for good crop establishment and, therefore, can impact yield even at this early stage. Hence, we suggest incorporating EV measurements into experiments by using the NDVI data in performance evaluations, such as stay-green trials. Our genomic analysis has identified QTL that is associated with EV, which are co-located or are closely linked to key genes controlling the plant development, such as plant height, coleoptile length, stay-green, and vernalisation. The results support the theory that EV is a trait regulated by pleiotropic genes. These findings may help identify the key drivers and determine potential trade-offs with important agronomic traits. Given that the trait is underpinned by many QTL with small effects, marker-assisted selection or gene-based approaches are likely to be challenging; however, genomic prediction approaches provide a suitable option for future breeding.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JC and LTH conceived the study. SV, SA, and JC performed the experiments. SV and AS analysed the phenotypic data. SV wrote the manuscript with further input from AS, RJS, JC, SA, and LTH. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.754439/full#supplementary-material

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3. Precision Phenotyping of Agro-physiological Responses and Water Use of Sorghum under Different Drought Scenario

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Precision Phenotyping of Agro-Physiological Responses and Water Use of Sorghum under Different Drought Scenarios

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Abstract: Understanding sorghum response to water stress at different developmental stages is important for developing sorghum varieties with improved tolerance to drought. This study set out to finely characterize key agro-physiological responses and water use of sorghum subjected to different drought scenarios. A greenhouse experiment was conducted using a DroughtSpotter facility that enables real-time quantification of water use by gravimetric tracking. Four different water treatments were assessed: (i) early vegetative drought stress (DS1), (ii) DS1 plus recovery (DS1R), (iii) late vegetative drought stress (DS2), and (iv) well-watered (WW). Plant pheno-morphology and yield data were recorded. Leaf fluorescence and photosynthesis were continuously recorded throughout the experiment. Our results showed that green leaf number and plant leaf area were more affected by DS2 than DS1 and delayed plant flowering. Nevertheless, plants in DS2 were taller and produced higher stem and leaf biomass compared to DS1. No significant difference was recorded in grain yield between DS1 and DS2 but were outperformed by DS1R and WW. The transpiration and photosynthetic rates were shown to decrease at the same time as the stomatal conductance. This can therefore be assimilated to a stomatal down-regulation limiting CO_2 uptake. However, the increase in intercellular CO_2 concentration is likely to indicate the presence of CO_2 in the substomatal cavity that was not conveyed to the carboxylation sites. This suggests a non-stomatal limitation of the photosynthesis. Moreover, the plants recovered quite well from DS1, and this was more prominent for physiological parameters than morphological ones. Globally, water use efficiency (WUE) for DS2 was higher compared to WW and DS1 treatments, confirming the growing point differentiation as a critical stage where drought stress should be avoided to ensure yield and better WUE. Adaptation responses were related to the reduction of transpiration through plant leaf area reduction, the reduction of stomatal conductance, and the increase of intercellular CO_2 limiting photosynthesis. Further studies focusing on the biomarkers of stress and transcriptomic analyses are needed to provide further insight into the drought adaptation mechanisms of this line.

Keywords: sorghum; drought adaptation mechanisms; photosynthesis; transpiration; water use efficiency; drought recovery

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1. Introduction

Sorghum is an important cereal crop in both dryland areas of Africa and India, where it is used as a staple crop for animal feed and as a source of income for small-scale farmers [1], and in Australia, the United States of America, and South America, where it is grown as a commodity crop for animal feed, bioethanol, or export. In contrast, in temperate Europe, it is a novel crop with minor importance that is mainly used for biogas production [2].

Sorghum as an annual crop can be grown in low rainfall environments, which makes it a good alternative to maize and sugarcane in these areas. Its genetic diversity and spectrum of adaptation to various cropping environments suggests considerable opportunity for improvement from the perspectives of diversifying end uses (in terms of biomass or grain) and adaptation to climate change [3]. As a C4 species, it has a high potential for producing biomass for different end uses depending on the range of biochemical composition available within its genetic diversity: digestible, poorly lignified biomass for biofuel, biogas, and forage, or lignified low sugar for bioplastic and bio-concrete [4,5]. Part of its value resides in this ability to be a multipurpose crop, which is a key advantage for optimizing land and resource uses and contributing to agriculture sustainability [6].

However, this sort of multipurpose production, which requires optimal access to light, water, and mineral nutrients, could be compromised when access to these resources is limited, mainly in the context of climate variability, which accentuates the scarcity of water resources. In particular, drought stress can negatively affect sorghum development and yield (e.g., biomass, grain) depending on its intensity and the stage at which it occurs. Drought persistence was shown to weaken sorghum plants and favor diseases or fungal infestation, leading to grain yield loss by 20 to 60% [7]. Occurrence of drought before anthesis can also reduce stem biomass production by 42% [8]. In some instances, drought causes a total failure of the crop [9].

Drought tolerance in sorghum involves the interaction between different morphological structures, biochemical expressions, and physiological functions [10]. Early drought stress is reported to induce changes in sorghum physiology by decreasing stomatal conductance, which can lead to the reduction of CO_2 uptake and the leaf transpiration rate [11]. It has also been shown to reduce leaf water potential and the maximum quantum efficiency of photosystem II [12,13]. However, the leaf area temperature was found to be increased under drought [14]. As a result, early drought stress affects plant photosynthesis and in fine biomass and grain production. Plant photosynthetic activity can also be disrupted when drought stress causes damage to cell membranes [15]. Therefore, genotypic ability to stabilize cell membranes is a promising way to enhance drought tolerance in crops [16]. Moreover, rewatering after drought stress contributes to recovering the performance of the plant. Gano et al. [11] researched a panel of ten West African sorghum varieties and reported good recovery for the number of appeared leaves and photosynthesis rate, whereas plant height and biomass production were hardly recovered. Jedmowski et al. [13] observed good recovery for leaf relative water content, whereas the recovery of leaf fluorescence parameters (Fv/Fm and performance index) were genotype-dependent. Moreover, Martínez-Goñi et al. [12] showed that plants prioritized the recovery of the net photosynthetic rate under ambient CO_2 compared to elevated CO_2. Therefore, since there is still a knowledge gap on sorghum recovery from drought stress after rehydration, there is a need for more investigation.

Understanding drought adaptation mechanisms is important for developing drought-tolerant sorghum varieties. The present work aimed at finely characterizing key agro-physiological responses and water use of sorghum subjected to different pre-flowering drought scenarios. The effect on plant structural growth and development, biomass, and grain production were assessed, as well as the anatomo-physiological mechanisms underlying drought adaptation in sorghum. In contrast to post-flowering drought stress in sorghum, which has been shown to be associated with stay green (e.g., Borrell et al. [17]), pre-flowering drought stress has received less attention. This may be due to the fact that post-flowering or terminal drought stress is of higher relevance globally, since at this stage, soil water tends to be depleted, while earlier vegetative growth in high-quality soils still benefits from stored water and is less likely to be drought-affected. However, at shallow soils with low water capacity (which are, e.g., typical sorghum cropping environments in temperate Europe), drought stress can occur at early stages. Pre-flowering and post-flowering drought tolerance in sorghum are believed to be based on different mechanisms [18], and usually, a sorghum genotype shows tolerance against only one of them [19]. Hence, the goal of this study was to provide more insight into the physiological responses to both of these drought stress regimes.

2. Materials and Methods

The current study was a greenhouse experiment conducted from September 2021 to April 2022 at the Department of Plant Breeding of Justus Liebig University of Giessen (Germany).

2.1. Plant Material

The sorghum genotype used was SC101, a conversion line [20] of caudatum–kafir race. It was chosen for this study from the sorghum collection of the Plant Breeding Department of Justus Liebig University of Giessen due to its observed superior drought tolerance in field experiments.

2.2. Methods

2.2.1. Experimental Conditions

The experiment was conducted as a full-growth cycle trial using the DroughtSpotter® system (Phenospex, Heerlen, The Netherlands) facility located at University of Giessen, Hesse, Germany. The DroughtSpotter® is a phenotyping platform designed for drought-stress-related experiments using growth containers placed on weight scales, which record weight deviations every five minutes throughout the whole experiment [21]. Further, every container is individually connected to an irrigation system, allowing specific irrigation treatment for each growth container of 60 L filled with 80 kg soil medium. The potting media were composed of 40% of excavated soil from a local field and 60% sand to ensure sufficient drainage throughout all soil layers. The soil texture and nutrient contents are presented in Table 1. The soil pH was 7. The soil texture was mostly sandy: fine sand (31.5%), middle sand (25.0%), large silt (20.6%), and clay (8.3%).

Table 1. Texture and nutrients contents of the soil used to fill the pots.

Analysis of Soil Texture			
Type	Size [mm]	Unit	Value
Fine sand	0.063–0.2	%	31.50
Middle sand	0.2–0.63	%	25.00
Large silt	0.02–0.063	%	20.60
Clay	<0.002	%	8.30
Middle silt	0.0063–0.02	%	7.50
Fine silt	0.002–0.0063	%	3.70
Large sand	0.63–2	%	3.50
Soil nutrient contents			
Element	Symbol	Unit	Value
Phosphorus	P_2O_5	[mg/100 g]	14.00
Potassium	K_2O	[mg/100 g]	8.00
Magnesium	Mg	[mg/100 g]	10.00
Iron	Fe	mg/kg	76.40
Copper	Cu	mg/kg	1.44
Zinc	Zn	mg/kg	1.75
Manganese	Mn	mg/kg	37.40
Boron	B	mg/kg	0.17
Molybdenum	Mo	mg/kg	<0.0150

2.2.2. Experiment Design and Management

A complete randomized design was used to test four water treatments, which included (i) early vegetative drought stress (DS1), (ii) DS1 plus recovery (DS1R), (iii) late vegetative drought stress (DS2), and (iv) well-watered treatment (WW). The experiment was conducted using two replications (i.e., containers per treatment). In each container, sorghum seeds were sown in three positions, which were located equidistant from each other. Three to four seeds were sown per position. At seedling stage, the plants were thinned to one per position, and three plants were maintained per container. Irrigation was provided every day at midnight following an automatic schedule.

Figure 1 depicts the cumulative transpiration showing how irrigation was managed for the different treatments. At physiological maturity, the cumulated water supplied to DS1, DS1R, and DS2 was 50%, 67%, and 61%, respectively, of the cumulated water supplied to the well-watered treatment (the control). In fact, DS1 was the first stress applied at 48 days after sowing, when the plants had 8 leaves and were at the growing-point differentiation stage (ca. BBCH 30; BBCH stands for *Biologische Bundesanstalt*, *Bundessortenamt und Chemische Industrie*, a scale used to identify the phenological stage of the plant). It was a decreased irrigation corresponding to 25% of field capacity. It lasted 63 days and thereafter, it was maintained at 35% of field capacity until physiological maturity. DS1R consisted of DS1 treatment, which after 63 days under stress (the plants had 15 leaves), was followed by optimal rewatering (70% of field capacity) until physiological maturity. DS2 was the treatment that was well-watered (70% of field capacity) until 118 days after sowing, and afterwards, irrigation was decreased (when the plants had 20 leaves) so as to maintain the treatment at 35% of field capacity until physiological maturity. As for the well-watered treatment, it was maintained under optimal irrigation (70% of field capacity) throughout the experiment.

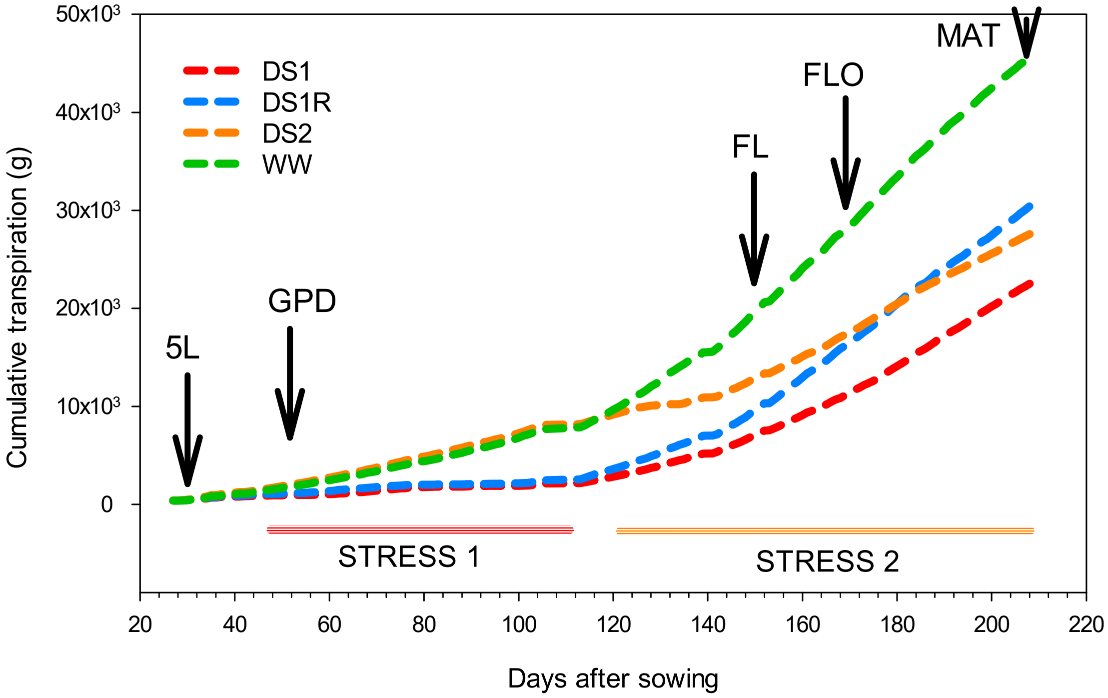

Figure 1. Cumulative transpiration compared between different water regimes. 5L: five leaf, GPD: growing point differentiation, FL: flag leaf, FLO: flowering, MAT: physiological maturity.

As for the fertilizer application, a WUXAL Super (AGLUKON Spezialduenger GmbH & Co. KG, Düsseldorf, Germany) nutrient solution was applied as recommended by the manual to exclude any nutrient deficiencies distorting the experiment. A volume of 50 mL was provided per position and 150 mL per container once a week. Fertilizer application started from the 20th day after sowing and was not applied when irrigation was withheld to impose stress so as to avoid additional water supply.

2.2.3. Weather Conditions

Temperature and relative humidity were recorded every ten minutes. Both parameters were used to compute the vapor pressure deficit (VPD) using the following formulae used by Alduchov et al. [22]:

$$VPD = SVP \times \left(1 - \frac{RH}{100}\right)$$

where SVP is the saturated vapor pressure, computed as:

$$SVP = 610.78 \times e^{\frac{-T}{(T+273.3)} \times 17.2694}$$

T is the temperature (°C), and RH is the relative humidity (%).

During the experiment, the temperature was 24.1–26.5 °C during the day and 18.1–23.8 °C during the night (Figure 2). These are moderate temperatures for sorghum, meaning that drought stress was not accompanied by high temperature stress, as it frequently occurs in its cropping environments. Inversely, the relative humidity was at 47.7–51.8% during the day and 48.4–55.3% during the night. As for VPD, it was 1.54 to 1.71 during the day and 0.93–1.53 during the night. The temperature remained almost similar over the duration of the experiment, while the relative humidity was fluctuating along the experiment.

The day/night regime was 16/8 h.

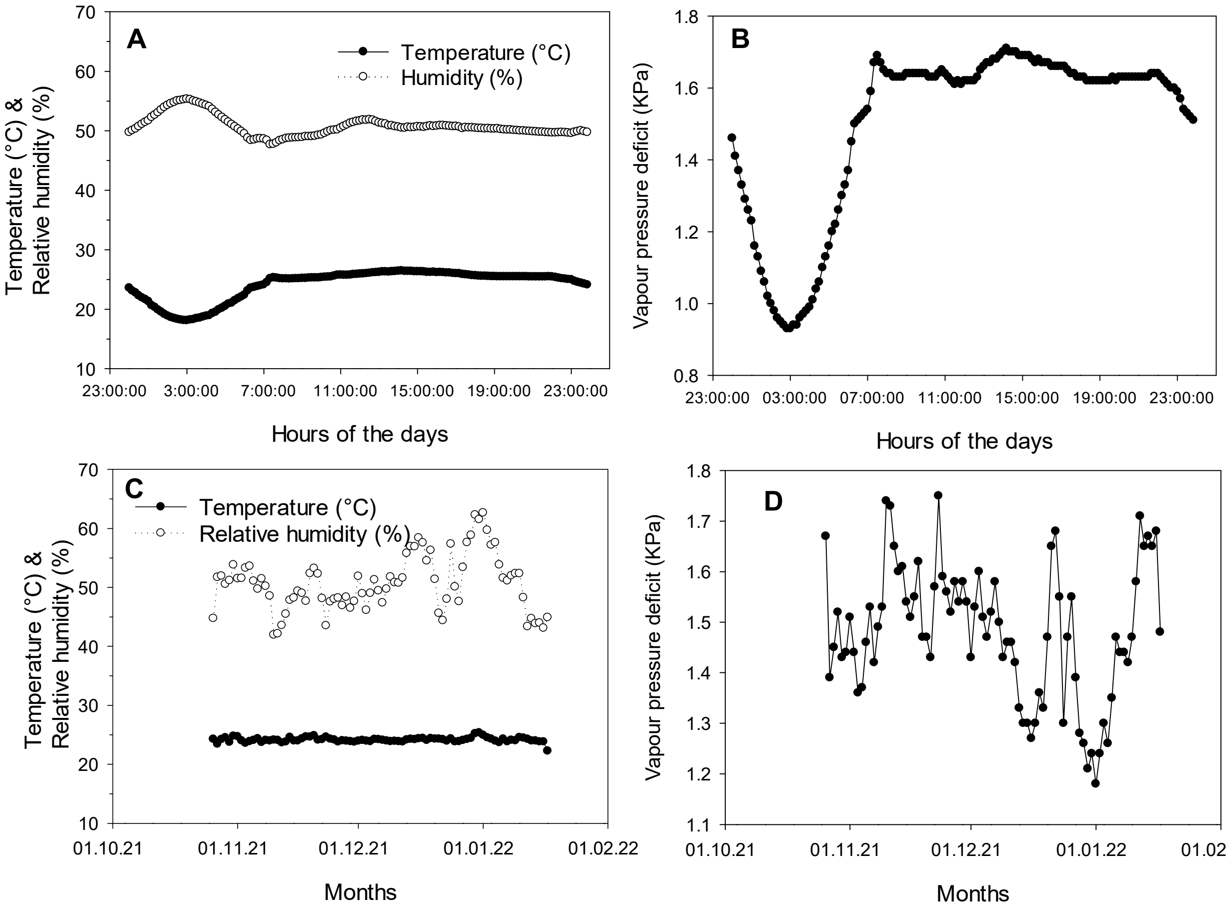

Figure 2. Daily averages of temperature and relative humidity (**A**), daily average vapor pressure deficit (**B**), and the temperature and relative humidity (**C**), and vapor pressure deficit (**D**) recorded along the experiment.

2.3. Data Collection

2.3.1. Plant Phenology and Morphology

At thinning, the three plants retained per container were tagged for dynamic characterization of plant growth and development. The appeared and ligulated leaves were counted three times per week. The green leaves were also counted. The leaf length and the largest part of leaf width were measured and were used to estimate the leaf area as the product of length, width, and allometric coefficient (0.69) [23]. Since the measurements were done thrice a week, the dimensions of all the leaves were taken up to the flag leaf. The total plant leaf area was computed as the sum of the area of the green leaves at the given stage. The phyllochron was computed as an inverse of development rate, calculated using the appeared leaf number as described in Tovignan et al. [24]. The phyllochron is the time that elapses between the appearance of two successive leaves.

Plant height was also measured dynamically up to flag leaf stage, as the height from the soil surface to the ligule of a given ligulated leaf. At physiological maturity (MAT), the plant height was also measured now from the soil surface to the tip of the panicle. The stem median diameter was measured at MAT using a digital Vernier caliper. The stem elongated internodes were counted. Peduncle length and panicle length and width were also taken at MAT.

The flag leaf and flowering are the phenological stages that were recorded. These stages were noted when 50% of the plants of each treatment reached the given stage.

2.3.2. Biomass Production

At physiological maturity, the three plants of each container were harvested and fresh stem and leaf weights were measured. The dry weights were estimated once stem and leaf biomass were dried in an oven at 70 °C during one week.

2.3.3. Grain Production

The panicle fresh and dry weight were recorded in grams in both control and stressed plants using a digital balance. The panicle fresh weight was measured immediately after harvesting of the plants. For the dry weight, the panicles were kept in oven for one week at 70 °C. Then, grain weight per panicle (GWP), one hundred seed weight (P100), and grain number per panicle (GNP) were taken.

2.3.4. Water Use Efficiency

Water use efficiency was estimated at physiological maturity as the ratio between the biomass produced and the cumulative transpiration. It was determined for stem fresh and dry weights, leaf fresh and dry weights, and grain production traits such as panicle fresh and dry weights and grain weight per panicle.

2.3.5. Gas Exchange, Chlorophyll Fluorescence, and Chlorophyll Content

Plant photosynthesis and leaf fluorescence were recorded three times per week from the setup of the first drought stress using LI-6800 (LICOR Biosciences, Lincoln, NE, USA). These measurements were taken on the third leaf from the top when the plants were photosynthetically active (between 9:00 a.m. and 1:00 p.m.), i.e., when the stomata are supposed to be well-opened, with the photon flux density fixed at 1000 µmol m^{-2} s^{-1}, and a mean temperature of 23 °C; the mean CO_2 concentration was 400 µmol mol^{-1} air, and the pump flow was 600 µmol s^{-1}.

Plant photosynthesis parameters such as net photosynthetic rate, transpiration rate, stomatal conductance (to water and CO_2), and intercellular CO_2 concentration were recorded. Instantaneous water use efficiency was calculated as the ratio between net photosynthetic rate and transpiration rate.

For the leaf fluorescence, a maximum yield of PSII (Fv/Fm) was used in this study.

Chlorophyll content was also measured three times per week using a hand-held chlorophyll content meter (CCM 200 plus, Opti-Sciences, Hudson, NH, USA).

2.3.6. Drought Recovery Index

Drought recovery index was calculated for the studied traits using the following formula:

$$\mathrm{DRI} = \log \mathrm{A} + 2 \log \mathrm{B}.$$

where A is the relative trait measured at the end of the drought and B is the relative trait measured 2 weeks after re-watering.

2.3.7. Leaf Anatomical Structures Analysis

In order to assess the percentage of cell membrane injury caused by drought stress, leaf samples were collected from the third leaf from the top, from well-watered and drought-induced treatments at the end of early vegetative drought stress. Sorghum leaf discs (~10 × 5 mm) were stained with AlexaFluor 488 phalloidin as previously described [25] with slight modifications [26]. The discs were fixed in 3.5% (*v*/*v*) formaldehyde in phosphate-buffered saline (PBS, pH 7.4) at room temperature overnight. After washing in PBS, the discs were immersed in 0.5% (*v*/*v*) Triton X-100 in PBS (pH 7.4) at room temperature overnight. The discs were then washed three times in PBS and stained with 0.66 mM AlexaFluor 488 phalloidin in PBS (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1 h in the dark before rinsing in PBS and mounting in PBS on glass slides. The interlocking marginal lobes were observed under a confocal microscope.

2.4. Data Analysis

One-way ANOVA analysis was conducted to test the effect of water regime on plant pheno-morphological and physiological data collected. The ANOVA was followed by a Tukey HSD test for mean comparison. Standard error was calculated to assess the degree of variation around means. ANOVA analysis and mean comparison test were performed using R 4.1.2 [27].

The leaf development rate of the plants in each water treatment was determined by assessing the slopes of the multi-regression lines between the appeared leaf number and the days after sowing. For this purpose, piecewise regression was used to detect the breakpoint at which the rate changed. Piecewise regression analysis was performed using SigmaPlot 14.0 (Systat Software, Inc., Los Angeles, CA, USA).

3. Results

3.1. Effect of Different Drought Scenarios on Plant Phenology

Drought stress applied has significantly affected plant phenology (Figure 3). The plants in drought conditions reached flag leaf (FL) and flowering (FLO) later than those grown under well-watered conditions. The flag leaf ligulation was delayed for DS1 and DS2 by 9 and 21 days, respectively, while the flowering time was delayed by 2 and 7 days for DS1 and DS2, respectively.

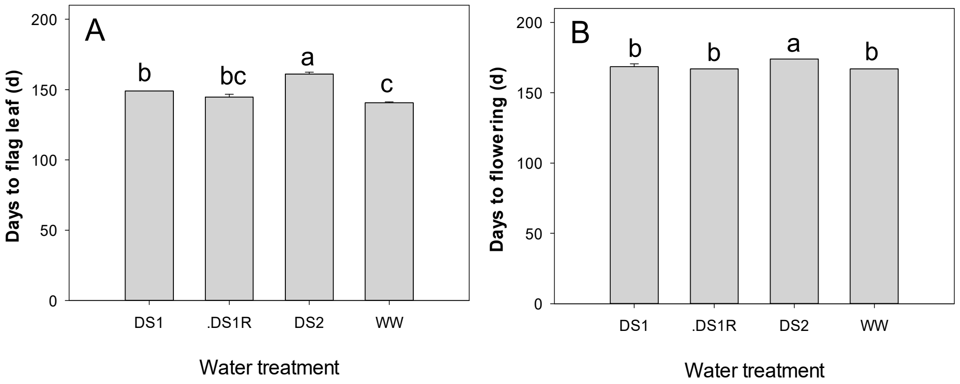

Figure 3. Days to flag leaf (**A**) and flowering (**B**) compared among the water treatments. Among water treatments, the traits mean with different letters are statistically different ($p < 0.05$).

3.2. Effect of Different Drought Scenarios on Plant Growth and Development

The effect of drought stress on plant morphology varied depending on the phenological stage. Plant height (PH) evolution compared among different water treatments (Figure 4E) revealed a 25% reduction for DS1 compared to well-watered treatment at 76 days after sowing. Once irrigation was resumed, the plants recovered from the first stress and showed a gain in height of 20%. After the induction of the second stress (DS2), a drastic reduction of 35% compared to well-watered (the control) was noticed on plant height at 149 days after sowing.

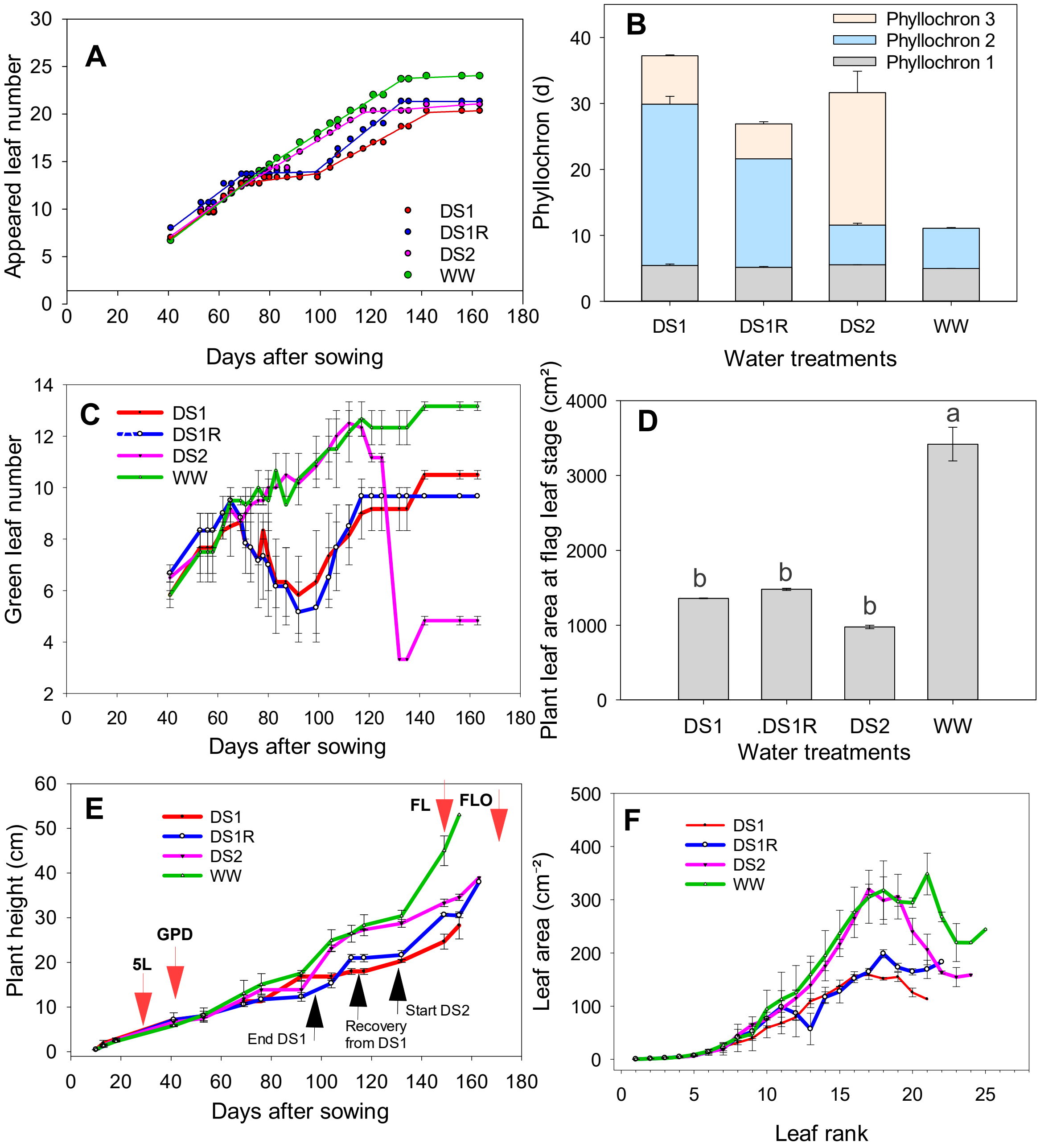

Figure 4. Leaf appearance rate (**A**), phyllochron (**B**), green leaves evolution (**C**), plant leaf area at flag leaf stage (**D**), plant height growth rate (**E**), and leaf area per leaf rank (**F**) compared among different water treatments. 5L: five leaf, GPD: growing point differentiation, FL: flag leaf stage, FLO: flowering. For plant leaf area at flag leaf stage, among water treatments, the mean values with different letters are statistically different ($p < 0.05$).

The development rate computed using the appeared leaf number showed three phases for the droughted treatments versus two for the well-watered treatment (Figure 4A,B). At the start, in the absence of any stress, a much faster first phase was observed for all the treatments, where the plant took an average of 5 days to produce a leaf. When the first stress occurred, the leaf production rate was completely slowed down, and the plant took an average of 20 days to produce a leaf (DS1 and DS1R). When irrigation was resumed for DS1R, there was a resumption of growth with a slightly more accelerated rate of 5 days to produce a leaf. On the other hand, for DS2 treatment, which had a normal growth before the stress induction, the leaf emergence was completely slowed, and the plant took almost 20 days to produce and develop the remaining leaves. In contrast, the irrigated treatment showed only two phases: a much faster first phase, wherein the plant took 5 days to produce a leaf, and a slightly slow second phase, wherein it took 6 days to produce a leaf.

The leaf area of the different leaf rank estimated showed more increased leaf expansion under well-water conditions compared to droughted ones (Figure 4F). At the flag leaf stage, DS2 experienced severe drought stress, and this significantly affected its plant leaf area compared to well-watered treatment (WW) (Figure 4D). Late vegetative drought stress led to higher reduction of plant leaf area at the flag leaf stage than early vegetative drought stress. This reduction of the plant leaf area resulted from the reduction of green leaves (Figure 4C). The DS2 treatment, which had time to set up the majority of its leaves and therefore had a great need for water, had to get rid of the majority of its leaves to reduce transpiration. This suggests an adaptation strategy to cope with late vegetative drought stress.

The way early drought stress affected the plant morphology is shown on Figure 5, particularly by reducing plant height, appeared leaf number, and the size of the leaves.

Figure 5. Picture showing plants in well-watered (**A**) vs. early drought stress (**B**) conditions 38 days after drought stress induction.

3.3. Effect of Different Drought Scenarios on Sorghum Agro-Morphological Parameters Measured at Physiological Maturity

Among the plant morphological traits assessed, water regime has significantly affected stem diameter and plant height when comparing drought stress treatments to well-watered (Table 2). However, the effect was slightly more depressive with DS1 than DS2. The length of peduncle was also affected but the effect was more depressive with DS2.

Table 2. Plant morphology, stem and leaf biomass, and grain production at physiological maturity, compared among different water treatments.

	DS1	**DS1 R**	**DS2**	**WW**	***p*-Value**
Plant morphology					
Diam (mm)	6 ± 0.3 b	7.5 ± 1.3 b	9.1 ± 0.9 ab	11.7 ± 1.4 a	0.0164 *
PH (cm)	82.6 ± 7.6 b	94.3 ± 1.8 ab	82.7 ± 0.5 b	106.7 ± 0 a	0.027 *
IN	7.7 ± 0.5 a	6.7 ± 0.5 a	6.7 ± 1.4 a	8.2 ± 1.2 a	0.36
Lped (cm)	26.0 ± 1.4 a	29.3 ± 1.4 a	19.5 ± 0.7 b	23.3 ± 0.5 ab	0.0151 *
Lpan (cm)	17 ± 1.9 a	19.5 ± 0.2 a	18.2 ± 0.7 a	21.5 ± 0.7 a	0.0805
Wpan (g)	2.6 ± 3.7 a	3.3 ± 5.1 a	2.8 ± 2.2 a	4.1 ± 5.8 a	0.0805
Biomass					
SFW (g)	28.1 ± 6.1 b	43.7 ± 3.1 b	39.2 ± 1 b	71.6 ± 4.6 a	0.00928 **
LFW (g)	24.1 ± 0.8 b	31 ± 2.3 b	30.6 ± 1.3 b	49.9 ± 2.5 a	0.00411 **
SDW (g)	8.5 ± 2.1 b	12 ± 0.7 b	13.7 ± 0.4 b	23.2 ± 1.6 a	0.00874 **
LDW (g)	7.6 ± 0.4 b	9.3 ± 0.8 b	14.5 ± 2.2 ab	18.1 ± 1.6 a	0.0155 *
Grain production					
PFW (g)	13 ± 3.3 b	25 ± 0.8 ab	15.3 ± 1.8 b	34.2 ± 4.8 a	0.0265 *
PDW (g)	9.5 ± 3.1 b	18.1 ± 0.8 ab	10.9 ± 1.3 ab	25.1 ± 4.3 a	0.0155 *
GWP (g)	8.3 ± 1.9 c	15.5 ± 0.6 ab	10.2 ± 1.3 bc	19.8 ± 0.8 a	0.0119 *
P100 (g)	1.4 ± 0.1 a	1.9 ± 0.2 a	1.2 ± 0.1 a	1.6 ± 0.2 a	0.0178 *
GNP	575.6 ± 123.2 b	833.8 ± 44.1 ab	867 ± 178.2 ab	1248.5 ± 54.4 a	0.0521

* $p < 0.05$; ** $p < 0.01$. Diam: stem median diameter, PH: plant height, IN: internode number, Lped: length of peduncle, LPan: length of panicle, Wpan: width of panicle, SFW: stem fresh weight, LFW: leaf fresh weight, SDW: stem dry weight, LDW: leaf dry weight, PFW: panicle fresh weight, PDW: panicle dry weight, GWP: grain weight per panicle, P100: hundred-seed weight, GNP: grain number per panicle. Among water treatments, the traits mean with different letters are statistically different ($p < 0.05$).

Stem (FW and DW) and leaf (FW and DW) biomass were significantly affected by drought stress, but the effect was more detrimental with DS1. This led to the reduction of stem biomass by 62% with DS1 vs. 43% with DS2, while leaf biomass was reduced by 55% and 29% by DS1 and DS2, respectively.

As for grain production, the water regime significantly affected all the grain production traits (PFW, PDW, GWP, P100) except the number of grains per panicle (GNP). DS1 had the most detrimental effect on these traits compared to DS2. Panicle weight was reduced by 62% by DS1 vs. 56% by DS2. Grain weight per panicle was reduced by 58% by DS1 vs. 48% by DS2. P100 was reduced by 13% by DS1 vs. 25% by DS2, while the number of the grains per panicle was reduced by 54% by DS1 vs. 31% by DS2.

3.4. Effect of Early and Late Vegetative Drought Stresses on Sorghum Physiology

Gas Exchange, Chlorophyll Content, and Fluorescence

The plant photosynthetic parameters (gas exchange, chlorophyll content, and fluorescence) recorded during the experiment are presented in Figure 6. Overall, a net contrast can be observed between the well-watered and the droughted treatments. Similar trend was observed for the gas exchange parameters such as transpiration rate (Figure 6A), net photosynthetic rate (Figure 6B), and stomatal conductance (water and CO_2) (Figure 6C,D), and instantaneous water use efficiency (Figure 6F). During early drought stress, these traits dropped sharply from 72 to 100 days after sowing (das) before increasing again two weeks later with the rewatering. The decrease during early vegetative drought stress was up to −30% for transpiration rate, −43% for net photosynthetic rate, −31% for stomatal conductance to both water and CO_2, and −49% for instantaneous water use efficiency. Meanwhile, an increase of 43% was observed for intercellular CO_2 concentration (Figure 6E). A similar trend was observed under the late vegetative drought stress between 128 to 139 days, but to a lesser extent. The late vegetative drought stress led to a reduction of transpiration rate by up to −18%, photosynthesis rate by −28%, stomatal conductance (water or

CO_2) by −17%, and instantaneous WUE by −22%, while intercellular CO_2 concentration increased by 6.4%.

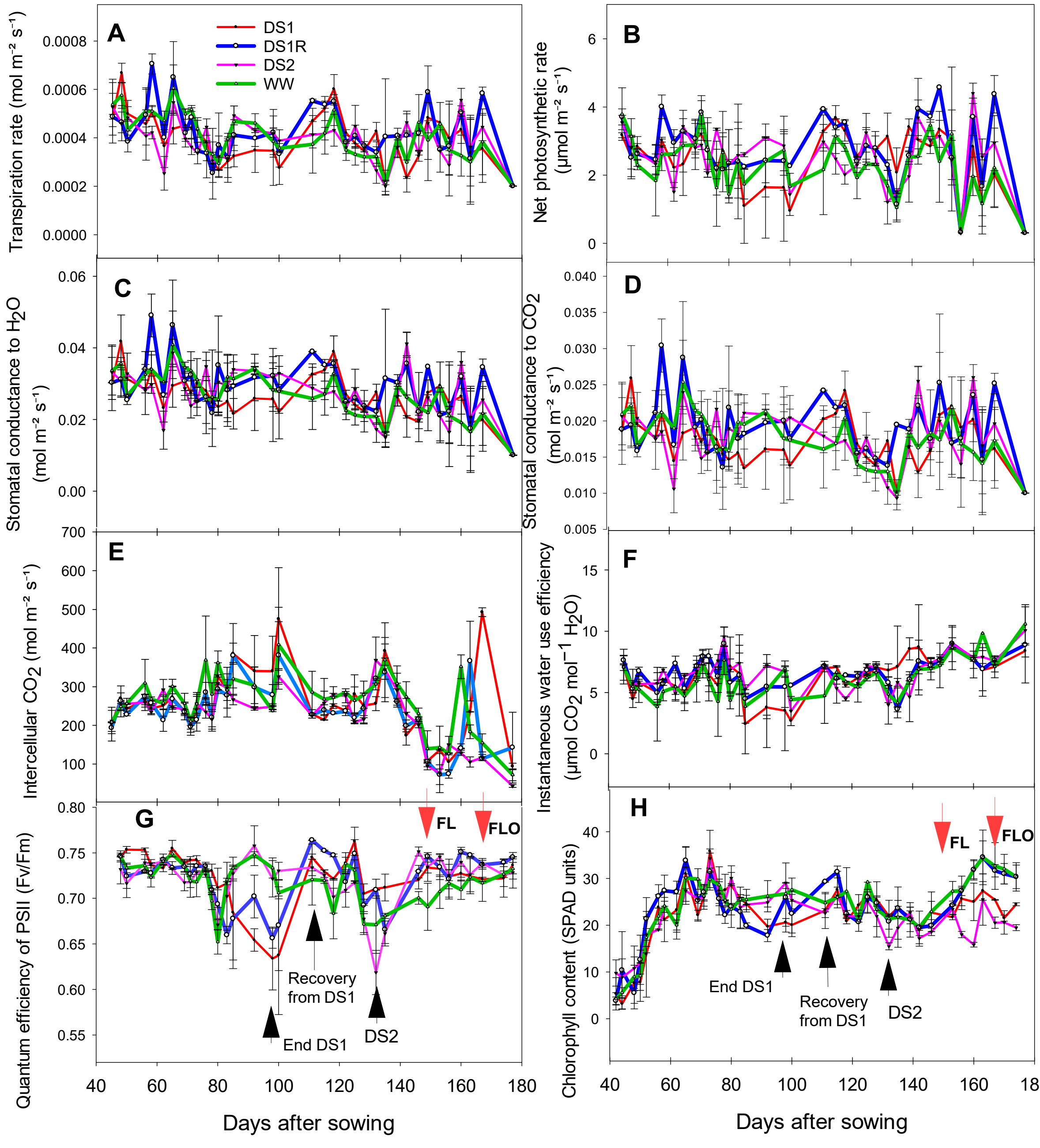

Figure 6. Transpiration rate (**A**), net photosynthetic rate (**B**), stomatal conductance to water (**C**), stomatal conductance to CO_2 (**D**), intercellular CO_2 (**E**), instantaneous water use efficiency (**F**), maximum yield of PSII (**G**), and chlorophyll content (**H**) recorded during the experiment, compared among different water treatments. Presented data are average value with standard error. FL: flag leaf stage, FLO: flowering.

As for leaf fluorescence, the maximum yield of PSII (Fv/Fm) was reduced by −14% from 80 to 92 days with the first drought stress applied and then increased again by 19% two weeks later with the rewatering. During the late drought stress, it was reduced by 14% from 125 to 132 days.

Chlorophyll content increased for all the treatments between 40 and 72 days. Along with the increasing of the first drought stress, it decreased by −26% between 69 and 73 days.

After rewatering, it increased again by 76% between 92 and 115 days. During the second drought stress, it decreased again by −28% between 125 and 132 days.

3.5. Drought Recovery Index and Water Use Efficiency

Table 3 presents the drought recovery index (DRI) estimated for plant morphological and physiological traits. The smaller the index value for a trait, the lower its ability to recover from drought stress. Traits with better recovery ability had values close to zero or slightly above. Plant height, number of appeared leaves, and intercellular CO_2 concentration showed a relatively low recovery ability with DRI of −0.36, −0.17, and −0.20, respectively. The other physiological traits of chlorophyll content, maximum yield of PSII (Fv/Fm), transpiration rate, stomatal conductance to CO_2, stomatal conductance to H_2O, instantaneous water use efficiency, and photosynthetic rate showed better recovery from drought stress, with DRI ranging from −0.04 to 0.52 as shown in Table 3.

Table 3. Drought recovery index of some morphological and photosynthetic traits.

	Drought Recovery Index (DRI)
Plant height, PH	−0.36
Number of appeared leaf	−0.17
Intercellular CO_2	−0.20
Vapor pressure deficit, VPD	−0.04
Chlorophyll content (SPAD)	−0.02
Leaf temperature	0.02
Maximum yield of PSII (Fv/Fm)	0.02
Transpiration rate	0.10
Stomatal conductance to CO_2, Gtc	0.32
Stomatal conductance to H_2O, Gsw	0.32
Instantaneous water use efficiency, iWUE	0.38
Photosynthetic rate	0.52

The water use efficiency (WUE) is one of the most functional indices that can be used to assess plant optimal water management and its ability to adapt to drought stress. WUE was calculated at physiological maturity as the ratio between the accumulated biomass and the recorded transpiration.

Table 4 presents the comparison of WUE among the different water treatments for stem and leaf biomass and grain production traits. The WUE depended on water regime and also on plant organs. The difference observed among water treatments was only significant ($p < 0.05$) for WUE of stem DW and leaf DW.

Table 4. Water use efficiency estimated for stem and leaf biomass and grain production at physiological maturity.

	DS1	**DS1R**	**DS2**	**WW**	***p*-Value**
Cumulative transpiration (Kg)	22.8 ± 3.2 a	30.8 ± 1.5 ab	27.8 ± 1.9 ab	46 ± 2.3 b	0.0486 *
Water use efficiency (g Kg^{-1})					
SFW	1.23 ± 0.19 a	1.42 ± 0.07 a	1.41 ± 0.03 a	1.56 ± 0.07 a	0.413
SDW	0.42 ± 0.02 ab	0.39 ± 0.02 b	0.49 ± 0.01 ab	0.52 ± 0.01 a	0.0277 *
LFW	1.06 ± 0.03 a	1.01 ± 0.05 a	1.1 ± 0.03 a	1.09 ± 0.04 a	0.552
LDW	0.33 ± 0.01 b	0.30 ± 0.02 b	0.55 ± 0.03 a	0.39 ± 0.03 ab	0.0163 *
PFW	0.2157 ± 0.1 a	0.81 ± 0.02 a	0.55 ± 0.05 a	0.74 ± 0.07 a	0.111
PDW	0.46 ± 0.05 a	0.59 ± 0.02 a	0.41 ± 0.02 a	0.55 ± 0.07 a	0.227
GWP	0.39 ± 0.03 a	0.5 ± 0.01 a	0.37 ± 0.03 a	0.43 ± 0.01 a	0.123

* $p < 0.05$. WUE: water use efficiency (g Kg^{-1}), SFW: stem fresh weight, LFW: leaf fresh weight, SDW: stem dry weight, LDW: leaf dry weight, PFW: panicle fresh weight, PDW: panicle dry weight, GWP: grain weight per panicle. Among water treatments, the traits mean with different letters are statistically different ($p < 0.05$).

As for grain production traits (PFW, PDW, GWP), no significant differences among the treatments were observed.

3.6. Early Drought Effect on Sorghum Leaf Anatomical Structures

The effect of early vegetative drought stress on the leaf anatomical structures is shown in Figure 7. The interlocking marginal lobe (IML) analysis shows that drought-stressed treatment has more IML compared to the well-watered one. In the zoomed area, IML under water stress is more packed in form and greater in number. The frequency of IML under drought stress was more increased compared to well-watered (Figure 7C).

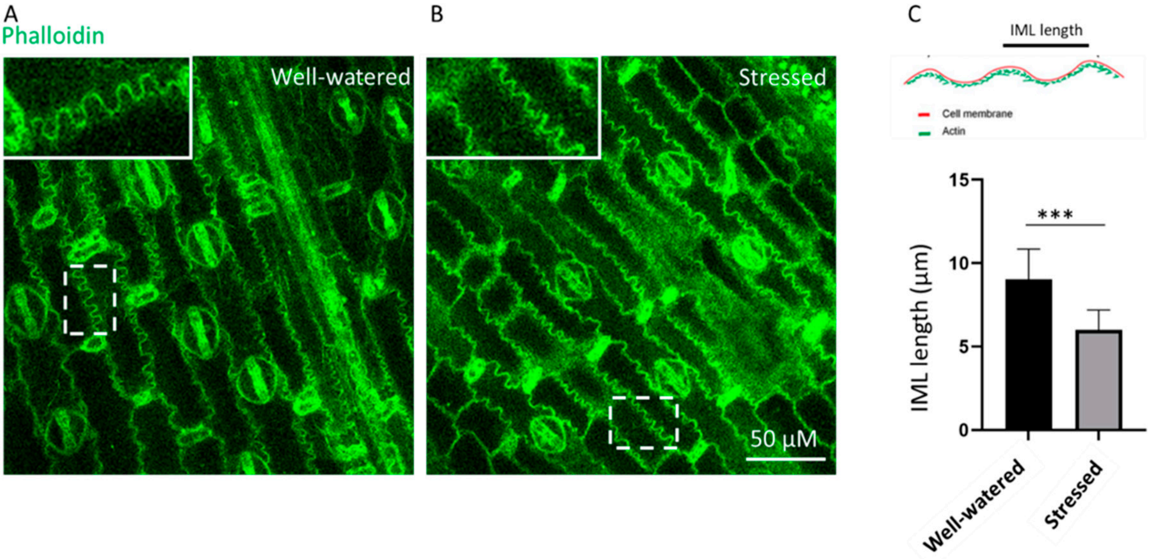

Figure 7. Microscopic images of phalloidin-stained sorghum from well-watered conditions (**A**) vs. early vegetative drought-stressed conditions (**B**). Quantification of the length of the IML in well-watered and drought-stressed sorghum (**C**). Data are means ± SD (n = 30 (10 cells)), *** $p < 0.001$.

4. Discussion

Drought stress, which is a growing concern in agriculture, can affect crop productivity depending on the development stage at which it occurs. Breeding for more drought-tolerant sorghum lines requires a better understanding of drought adaptation mechanisms (anatomo-physiological) deployed by sorghum depending on the drought scenario. This study analyzed sorghum adaptation mechanisms to early and late vegetative, and the recovery from early vegetative drought stress.

4.1. Drought Stress Effect on Plant Phenology, Growth and Development, and Production

In this study, plant phenology was affected through the delay of flag leaf ligulation and flowering. The plants in DS2 conditions, developed most of their leaves (17 out of 19 leaves) before induction of the late vegetative stress. As the presence of all these leaves increased the water requirement of the plant, occurrence of drought stress at this stage was fatal and led to cessation of the plant growth and development, particularly the emergence and development of the very last leaves, which took long to achieve. On the other hand, the plants in DS2 conditions rapidly lost most of their green leaves (an average of 5 leaves for DS2 vs. 13 for WW, remained at flag leaf stage) which could be an adaptation strategy to limit water loss through transpiration. In previous studies, plant phenology, mainly the panicle initiation and flowering time, was also shown to be strongly affected by drought stress [28]. These authors observed with a late vegetative drought stress an increase of the days to flowering and a reduction of the rate of panicle development. This influence on plant phenology was also shown on 21 sorghum genotypes by Rakshit et al. [29], who observed an increase of the days to anthesis under drought stress that was genotype-dependent. In contrast, plant growth and development, above-ground biomass, and grain production were more affected by early drought stress with a decrease of 62% for stem biomass, 55% for leaf biomass, and 62% for panicle dry weight. Previous studies have also reported a decrement of plant height, above-ground biomass and grain production under early vegetative drought stress conditions [11,30,31]. In this study, the fact that the grain number per panicle was more affected by early vegetative drought compared to late vegetative drought could be explained by the fact that panicle growth starts very early inside the shoot from the growing point differentiation onwards. However, the hundred-seed weight was much more affected by late vegetative drought stress and could be a source limitation for grain filling.

4.2. Drought Stress Effect on Plant Physiology

Our results showed that the effect of drought stress was more detrimental on all the physiological parameters during the early vegetative stage compared to the late vegetative stage. The drought stress decreased transpiration and net photosynthetic rates. Meanwhile, the stomatal conductance was also decreased. This could possibly be due to the lowering of CO_2 availability for photosynthesis, as a consequence of stomata closure. Many studies reported inhibition of plant photosynthesis due to stomata closure under drought stress to limit water loss through transpiration [32–35]. On the other hand, an increase of intercellular CO_2 concentration (Ci) was observed for the droughted treatments, indicating the presence of CO_2 in substomatal cavities. However, the CO_2 present would not be transferred to the carboxylation sites in the chloroplasts and could explain the inhibition of the photosynthesis activity, which was non-stomatal limitation but appeared to be a mesophyll conductance. Many previous studies reported a non-stomatal limitation of the photosynthesis activity on sorghum, maize, and medicago [36–38]. Mesophyll conductance is reported in many studies to be responsible for carbon fixation reduction in many crops [39–41]. Fv/Fm is used in many studies as index to assess the resistance of the crops to drought stress [37,42,43]. In the present study, the early and late vegetative drought stresses led to the reduction of the maximum yield of PSII (Fv/Fm). Fv/Fm was shown in previous studies to be reduced under drought stress in sorghum [15,44]. The decrement observed in this study could indicate damage caused to the photosystem II reaction center, mainly to the thylakoid membranes. Additionally, a decrease of 14% was also observed for chlorophyll content during early and late vegetative drought stresses. This result is in line with other studies showing negative impact of drought stress on leaf chlorophyll content [15,43,45]. This decrease could indicate chlorophyll degradation due to the damage to the chloroplasts and thus limit photosynthesis [46].

Moreover, plants can develop some protective structures to strengthen their cell wall to cope with drought stress. In previous studies, interlocking marginal lobe (IML) formation was shown to be a drought-tolerance-associated feature [47,48]. In the present study, IML formation was found in both well-watered and drought-stressed sorghum, because sorghum is known for its drought tolerance. In addition, the frequency of the IML increased under drought stress, which again confirmed its role in drought tolerance.

4.3. Sorghum Water Use Efficiency and Ability to Recover from Early Drought Stress

Many studies have shown that water use efficiency (WUE) is an important functional index related to plant growth and productivity, and it is used to determine crop optimal water management [49]. In the present study, the WUE differed depending on the plant organ (stem, leaf, grain) from one water treatment to another. WW treatment showed the highest WUE for SFW and SDW, and DS2 better maintained WUE for stem biomass than DS1. For the leaf biomass, DS2 showed the best WUE. As for grain production traits (PFW, PDW, GWP), the highest WUE was obtained with DS1R; however, these differences were not significant. DS2 seemed to better maintain WUE for grain production traits than DS1. These results suggest that the 70% of field capacity applied for the WW treatment was beyond what was needed for optimal WUE; however, the goal of the WW treatment was rather to show the yield potential under non-water-limited conditions. These results are consistent with Bhattarai et al. [50], who studying three irrigations treatments (I_0 = 50 mm, I_1 = 200 mm, I_2 = 350 mm), found that for above-ground biomass at maturity, I_0 was followed by I_1, which reached higher WUE than I_2. Similar result was also obtained by Abdel-Motagally [51], who studied three water regimes with three grain sorghum genotypes, found that the sorghum plants that received the lower water supply obtained the higher WUE, contrary to those that received the higher water supply. Moreover, Mastrorilli et al. [52], studying a sweet sorghum cultivar named Keller, subjected to early and late vegetative drought stress, found that WUE for the late vegetative stage resulted in higher WUE than well-irrigated and early vegetative drought stress. In short, our results showed the growing point differentiation as a stage at which it is important to avoid drought stress to the plant in order to avoid yield loss and decrease in WUE. Many other studies have reported the growing point differentiation as a critical stage at which drought stress should be avoided and recommended fertilization application prior to this stage and irrigation supply during this stage to increase grain production, especially the number of seeds per head, which is established shortly after this stage [53,54].

Moreover, the recovery test performed in this study showed that the physiological traits such as chlorophyll content, maximum yield of PSII (Fv/Fm), transpiration rate, stomatal conductance to CO_2, stomatal conductance to H_2O, instantaneous water use efficiency, and photosynthetic rate presented good recovery from early drought stress compared to morphological traits such as plant height and leaf number, which showed low DRI. This result confirms those obtained by Gano et al. [11], who studied early vegetative drought stress on a panel of ten West African sorghum genotypes and showed good recovery index for physiological parameters contrarily to morphological parameters such as plant height and above-ground biomass. They related this photosynthesis recovery ability to the fact that the photosystem reaction center was not irreversibly affected by oxidative damage and also to the plasticity of the genotype to resume with photosynthesis upon rehydration. This reasoning was also given by Devnarain et al. [55] in their study of five African sorghum varieties, which were able to maintain chlorophyll and carotenoid levels upon rehydration after drought stress. Moreover, Martínez-Goñi et al. [12], studying the ability of sorghum to adapt to drought combined with elevated and ambient CO_2, observed that after being subjected to drought, sorghum prioritized recovery of its photosynthesis activity upon rehydration mainly by rapidly opening its stomata and increasing the transpiration rate. They also observed that sorghum required more than 7 days of rehydration to fully recover from drought stress.

5. Conclusions

Our results show that early vegetative drought was more detrimental on plant vegetative growth, development, and biomass than late vegetative drought stress. Green leaf number and plant leaf area were found to be more affected by DS2 than DS1, and this resulted in delaying the flowering time. The reduction of plant leaf area observed for late vegetative droughted plants is likely an adaptation strategy to limit water loss through transpiration. Nevertheless, plants in DS2 were taller and produced higher stem and leaf biomass compared to DS1. Grain yield was similar for DS1 and DS2, but were outperformed by DS1R and WW. Early drought stress was found to be more deleterious on all the physiological parameters than late vegetative drought stress. The transpiration and photosynthetic rates were shown to decrease at the same time as the stomatal conductance, while an increase of intercellular CO_2 concentration limited CO_2 uptake and transfer to carboxylation sites to allow photosynthesis. Moreover, the plants recovered quite well from DS1 by increasing the photosynthesis parameters. The WUE for the late vegetative droughted treatment resulted in higher WUE compared to well-irrigated and early vegetative drought treatments. Therefore, it seems important to avoid drought stress at the early vegetative stage (growing point differentiation) where the plants are in rapid growth and development to avoid yield loss and decrease in WUE.

The leaf area reduction to limit transpiration, the reduction of stomatal conductance, and the increase in intercellular CO_2 concentration are the adaptative responses observed in this line. Studying some stress biomarkers and transcriptomic profile will provide further insights into its drought adaptation mechanisms.

Author Contributions: T.K.T., S.W., S.V. and R.S. designed the original research questions. T.K.T., Y.B., S.M.A. and S.V. performed the experiments and collected the data. T.K.T. analyzed the data with support from all co-authors. T.K.T. led the writing of the manuscript with contributions from all authors. All authors have read and agreed to the published version of the manuscript.

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4. Effect of a QTL on wheat chromosome 5B associated with enhanced root dry mass on transpiration and nitrogen uptake under contrasting drought scenarios in wheat

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Effect of a QTL on wheat chromosome 5B associated with enhanced root dry mass on transpiration and nitrogen uptake under contrasting drought scenarios in wheat

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Abstract

Background A sufficient nitrogen supply is crucial for high-quality wheat yields. However, the use of nitrogen fertilization can also negatively influence ecosystems due to leaching or volatile atmospheric emissions. Drought events, increasingly prevalent in many crop production areas, significantly impact nitrogen uptake. Breeding more efficient wheat varieties is necessary to achieve acceptable yields with limited nitrogen and water. Crop root systems play a crucial role as the primary organ for absorbing water and nutrients. To investigate the impact of an enhanced root system on nitrogen and water use efficiency in wheat under various irrigation conditions, this study conducted two experiments using precision phenotyping platforms for controlled drought stress treatment. Experiment 1 involved four contrasting winter wheat genotypes. It included the Chinese variety Ning0604, carrying a quantitative trait locus (QTL) on chromosome 5B associated with a higher root dry biomass, and three elite German varieties, Elixer, Genius, and Leandrus. Experiment 2 compared near-isogenic lines (NIL) of the three elite varieties, each containing introgressions of the QTL on chromosome 5B linked to root dry mass. In both experiments, nitrogen partitioning was tracked via isotope discrimination after fertilization with 5 Atom % ^{15}N-labeled KNO_3^-.

Results In experiment 1 the quantification by ^{15}N isotope discrimination revealed significantly ($p < 0.05$) higher nitrogen derived from fertilizer in the root organ for Ning0604 than those of the three German varieties. In experiment 2, two out of three NILs showed a significantly ($p < 0.05$) higher uptake of N derived from fertilizer than their respective recipient line under well-watered conditions. Furthermore, significantly lower transpiration rates ($p < 0.1$) were observed in one NIL compared to its respective recipient.

Conclusions The combination of the *DroughtSpotter* facility coupled with ^{15}N tracer-based tracking of N uptake and remobilization extends the insight into the impact of genetically altered root biomass on wheat NUE and WUE under different water availability scenarios. The study shows the potential for how a modified genetic constitution of the locus on wheat chromosome 5B can reduce transpiration and enhance N uptake. The dependence of the observations on the recipient and water availability suggests a need for further research to investigate the interaction with genetic background traits.

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Keywords ^{15}N-Tracer, Nitrogen Derived from Fertilizer, Nitrogen Uptake Efficiency, Water Use Efficiency, Drought Stress Tolerance

Background

The increasing world population and simultaneously occurring environmental changes resulting from climate change are creating an urgent need for sustainable agriculture, which will not only allow the production of sufficient food but also cause reduction in the environmental footprint of agricultural production [1, 2]. Nitrogen (N) is the most important plant nutrient used as a fertilizer in terms of quality and quantity in modern wheat production and thus essential for farmers to meet their economic goals [3]. Furthermore, N is the key nutrient for high quality baking wheat, since the expression of the protein fractions gliadin and glutenin is strongly affected by the N supply in addition to the genetic background [4, 5]. Consequently, to ensure a sufficient protein production farmers usually apply three N applications to ensure a high supply of N [6]. However, overuse of N fertilizer can also have negative environmental impacts, such as the high use of fossil fuel and the release of carbon dixoide (CO_2) during the artificial synthesis of ammonia, which requires approximately 2% of the world's energy [7, 8]. Furthermore, direct greenhouse gas emissions resulting from ammonia and nitrous oxide are major contributors to climate change, and the leaching of nitrate contributes strongly to agroecosystem groundwater contamination [9–12]. Anthropogenically induced climate change has also increasingly impacted crop production due to intense, erratic drought stress events. Drought stress is one of the most relevant abiotic stress factors, especially in arid and semi-arid cropping regions, where it can cause yield losses ranging from 13 to 94% [13, 14]. As a result of these challenges, there is a growing interest in breeding varieties that have higher N use efficiency (NUE) and water use efficiency (WUE), thereby maintaining the required yield and quality parameters while reducing the environmental footprint [15]. NUE is defined as grain yield per unit of available N in the soil, or, concerning biomass production, as fresh matter or dry mass per unit of available N in the soil [15–17]. WUE is defined as grain yield or biomass per unit of water used [18].

NUE is a complex trait that can be described by two main components: N uptake efficiency (NupE) and N utilization efficiency (NutE). NupE describes the ability of the plant to mobilize and acquire N from the soil, whereas NutE describes the ability of the plant to translocate the acquired N into yield-producing organs (seed or biomass) [17, 19]. Especially under low N availability, high NupE is a critical variable that can most effectively increase total NUE in wheat [20, 21]. This is also in line with studies that found that NUE under low N availability is mainly explained by high NupE rather than NutE [22]. Conversely, the importance of NutE for overall NUE increases with increasing N availability, hence the NupE is particularly important in intensive cropping systems.

Roots, as the primary organ of nutrient and water uptake, are of particular significance to increase NupE and NUE for intensive wheat production systems [23–25]. For example, a root system with an increased root length density is considered to have a specifically high potential to increase NupE [26]. Particularly the mobilization of N before anthesis contributes more to N storage in the grain than remobilization after anthesis [22]. A rapid and extensive root system development in early growth stages can increase the pre-anthesis N uptake and thus increase the total NUE [27]. Therefore, it is especially important to investigate the influence of an increased root system on the mobilization (NupE) as well as the translocation (NutE), to obtain a holistic picture for the establishment of NUE. For this purpose, the use of ^{15}N-labeled fertilizers is particularly advantageous to track the utilization of applied N beginning from a defined developmental stage [28].

Due to the focus on aboveground traits in crop breeding programs, it is assumed that modern elite varieties may have undergone an unintended negative selection against advantageous root traits at the cost of aboveground biomass and yield [29]. One reason for this might be the pleiotropic effects of favorable aboveground traits which have a negative effect on root characteristics. For example, negative pleiotropic effects on root traits have been demonstrated in several reduced height genes, which have played a major role in the improvement of harvest index in modern elite varieties [30]. Linkage drag may also lead to unfavorable root systems in modern elite cultivars. For example, it was found that two haplotype blocks containing quantitative trait loci (QTL) with positive effects on root dry mass (RDM) were absent in European elite wheat cultivars due to linkage drag with a QTL controlling heading date [31]. The two beneficial haplotype blocks, *Hap-5B-RDMa* and *Hap-5B-RDMb*, were found to be highly conserved and exclusively present in Chinese wheat varieties.

To date, most modern wheat breeding programs do not apply specific selection for genotypes with positive

Table 1 Haplotype variants for *Hap-5B-RDMa* and *Hap-5B-RDMb* within the plant material used in experiment 1 alleles marked in bold letters represent the haplotype variant associated with an enhanced root growth

	Hap-5B-RDMa		**Hap-5B-RDMb**		
	BobWhite_c43_86		**BS00029852_51**	**Tdurum_contig48959_1172**	
Genotype	**Allele**	**Haplotype Variant**	**Allele**	**Allele**	**Haplotype Variant**
	[G/A]		[C/T]	[G/A]	
Elixer	GG	H1	CC	GG	h1
Genius	GG	H1	CC	GG	h1
Leandrus	**AA**	**H2**	TT	GG	h2
Ning 0604	**AA**	**H2**	**TT**	**AA**	**h3**

root traits, in large part due to the difficulty of phenotypic quantification [32]. One way to overcome this problem could be the use of marker-assisted selection for root-associated QTL, provided such QTL have been sufficiently tested and their positive effects validated. Here the impact of the haploblock types *Hap-5B-RDMa* and *Hap-5B-RDMb* was tested with respect to: (i) water use, (ii) N uptake and utilization, and (iii) their influence on aboveground plant organs, to offer potential solutions for practical plant breeding to improve water and nutrient use through altered root system in modern wheat lines.

Methods

Plant material

Experiment 1

In experiment 1 a set of four genotypes was investigated. These comprise the Chinese wheat variety Ning0604, which carries the two root-associated haplotype blocks *Hap-5B-RDMa* and *Hap-5B-RDMb* described in previous studies [31]. The remaining three varieties were the German elite winter-wheat varieties Elixer, Genius and Leandrus (recipients) respectively. Each elite variety corresponds to a specific wheat grain quality group. Elixer is characterized as a C-group wheat variety according to the German quality classification system, producing seeds with lower protein concentrations. Genius is listed within the E-group, which has the highest standards for protein content and quality. Leandrus is classified as a regular baking wheat variety (A-group). Leandrus carries one RDM haplotype block allele (*Hap-5B-RDMb*) but not the other, whereas the two other elite varieties do not carry either of the haplotype block alleles under investigation in this study (Table 1).

Experiment 2

To examine the isolated effect of the root-associated haplotype-blocks, near-isogenic lines (NIL) were developed by crossing each of the three recipients with the Ning0604 and subsequent marker-assisted backcrossing over three generations. After each backcrossing step, foreground selection on the root-associated haplotype-blocks was performed using three SNP markers associated with *Hap-5B-RDMa* and *Hap-5B-RDMb*, while background selection on the respective recipient was performed using 29 SNP markers (Table 2). For experiment 2, three NILs were selected, corresponding to the three elite recipients and carrying both haplo blocks of interest in the largest possible genetic background of each recipient. In addition, the three corresponding recipients (PAR) were also tested again, so that experiment 2 consists of six genotypes.

Phenotypic analysis

Plant cultivation and experimental setup

To gain a detailed view of WUE, transpiration efficiency, NUE as well as growth behaviour under near-field conditions, experiment 1 and experiment 2 were conducted using two different custom-built *DroughtSpotter*® precision phenotyping systems (Phenospex, Heerlen, Netherlands). The *DroughtSpotter*® is a phenotyping platform designed for drought-stress related trials using large growth containers placed on gravimetric scales, which record weight deviations every five minutes throughout the whole experiment. Every container weight scale is also individually connected to an irrigation system, allowing specific irrigation treatments for each container. The large containers enable multiple plants to be grown at field planting density to simulate field growing conditions with canopy and underground nutrient competition.

Experiment 1 was conducted as a full growth cycle trial using the *DroughtSpotter XXL* (DS XXL) foil house facility located at the Rauischholzhausen research facility of

Table 2 SNP markers used for NIL development alleles. Underlined alleles represent the undesired allele frequency for the respective background

Marker	Allele	Background	Genotype		
			Elixer	**Genius**	**Leandrus**
SNP-01	[C/T]	Ning0604	T:T	T:T	T:T
SNP-02	[C/T]	Ning0604	T:T	T:T	T:T
SNP-03	[G/A]	Ning0604	A:A	A:A	A:A
SNP-04	[G/A]	Parental	A:A	A:A	A:A
SNP-05	[G/A]	Parental	A:A	A:A	A:A
SNP-06	[G/A]	Parental	G:G	G:G	G:G
SNP-07	[G/A]	Parental	G:G	G:G	G:G
SNP-08	[C/T]	Parental	C:C	C:C	C:C
SNP-09	[C/T]	Parental	C:C	C:C	C:C
SNP-10	[C/T]	Parental	T:T	T:T	T:T
SNP-11	[G/A]	Parental	G:G	G:G	G:G
SNP-12	[G/A]	Parental	A:A	A:A	A:A
SNP-13	[G/A]	Parental	A:A	A:A	A:A
SNP-14	[C/T]	Parental	T:T	C:C	C:C
SNP-15	[G/A]	Parental	A:A	A:A	A:A
SNP-16	[C/T]	Parental	C:C	C:C	C:C
SNP-17	[G/A]	Parental	A:A	A:A	A:A
SNP-18	[C/T]	Parental	C:C	T:T	T:T
SNP-19	[G/A]	Parental	A:A	A:A	A:A
SNP-20	[G/A]	Parental	G:G	G:G	G:G
SNP-21	[C/T]	Parental	C:C	C:C	C:C
SNP-22	[C/T]	Parental	C:C	C:C	C:C
SNP-23	[C/T]	Parental	C:C	C:C	C:C
SNP-24	[G/A]	Parental	A:A	A:A	A:A
SNP-25	[C/T]	Parental	C:C	C:C	C:C
SNP-26	[G/A]	Parental	A:A	A:A	A:A
SNP-27	[G/A]	Parental	A:A	A:A	A:A
SNP-28	[G/A]	Parental	A:A	A:A	A:A
SNP-29	[C/T]	Parental	C:C	A:A	C:C
SNP-30	[G/A]	Parental	A:A	A:A	A:A
SNP-31	[G/A]	Parental	G:G	G:G	G:G
SNP-32	[G/A]	Parental	A:A	A:A	A:A

the Justus Liebig University Giessen in Hesse, Germany. The DS XXL is a semi-controlled phenotyping platform comprising a total of 240 large plant containers (90 L volume) placed on individual scales. Each container was filled with 150 kg of a soil mixture composed of 40% excavated soil from a local field and 60% sand to ensure sufficient drainage throughout all soil layers (Fig. 1A and B). Climate conditions including temperature (°C), relative humidity (%), and photosynthetically active radiation (µmol/m^2) were recorded throughout the whole experiment from a weather station positioned in the center of the greenhouse. In experiment 1, the records of climate data, for the period from -84 days after Heading (DAH) until 28 DAH, show an average temperature of 13.41 °C and a mean relative humidity of 62.94%. Further information regarding the environmental conditions is given in Table 3 and Additional file 1. Containers were sown in three rows of 21 seeds per row and rows were thinned after germination to achieve a total plant density of 48 plants per container.

In experiment 1, three different irrigation treatments were applied for each genotype. A well-watered treatment was set at 60% field capacity during the whole duration of the trial and was used as a control. Further, two drought treatments were used to investigate the drought stress resistance, varying in the date of application. The first treatment, drought scenario 1, was applied at the heading date (0 DAH) and the second treatment, drought scenario 2, was applied 14 days after heading (14 DAH). For each drought treatment, 40% field capacity was used. Each treatment was replicated three times as a fully randomized complete block design containing one replication per block.

Experiment 2 was conducted under controlled conditions using the *DroughtSpotter L* (DS L) in a greenhouse facility located at the Justus Liebig University Giessen. The DS L uses a total of 48 large plant containers (60 L volume) placed on individual heavy-duty scales. Each container was filled with 80 kg of the same soil mixture used in the DS XXL (Fig. 1C and D). Containers were sown with 21 seeds per container arranged in concentric circles and thinned after germination to achieve a total plant density of 16 plants per container. Climate conditions including temperature (°C) and relative humidity (%) were recorded using three data loggers (EL-USB-2, Lascar Electronics, Whiteparish, UK) placed throughout the greenhouse chamber. The air temperature in experiment 2 was set to 24 °C during the day and 18 °C at night, however, fluctuations in temperature could occur due to intense solar radiation, resulting in higher temperatures observed at times. In experiment 2, the records of climate data show an average temperature of 24.25 °C and a mean relative humidity of 50.24%. Further information regarding the environmental conditions is given in Table 3 and Additional file 1. Day length was set on a long day interval with 16-h days and 8-h nights. To ensure sufficient radiation, high-pressure sodium-vapour lamps (SOD Agro 400–230, DH Licht GmbH, Wülfrath, Germany) were used as soon as sunlight radiation dropped below 12 klx. Two irrigation treatments were used in experiment 2, which began 21 days after sowing (0 DAT) and were kept constant during the whole duration of the trial. A well-watered control was set at 60% field capacity and drought treatment at 40% field capacity. Each treatment was replicated three times. The experiment was laid out as a fully

Fig. 1 General Setup of the DroughtSpotter® phenotyping platforms located at the University of Giessen (**A**) 90L growth container placed on weights scale in *DroughtSpotterXXL* with 48 wheat plants sown in three rows á 16 plants (**B**) Impression of the full *DroughtSpotterXXL* test facility (**C**) 60L growth container placed on weight scale in *DroughtSpotterL* with 16 plants (**D**) Impression of the full *DroughtSpotterL* test facility

Table 3 Climatic conditions for experiment 1 and experiment 2

Experiment		Temperature [°C]	Rel. Humidity [%]	PAR [µmol/m^2]
Experiment 1	Mean	13.41	62.94	276.13
	Median	13.8	60.31	272.69
	SD	5.15	11.54	97.10
	Min	3.42	40.85	67.54
	Max	24.22	92.78	461.62
Experiment 2	Mean	24.25	50.24	NA
	Median	25.0	49.5	NA
	SD	3.01	6.83	NA
	Min	15.0	25.5	NA
	Max	37.0	76.0	NA

randomized complete block design, containing one replication per block.

Use of ^{15}N-Tracers

Application and sample collection

In experiment 1, N was applied at the end of the booting stage (Zadoks 57–59). Each container received 2 g Nin the form of 5 atom % $K^{15}NO_3^-$. Samples were taken four times at 0 DAH, 14 DAH, 21 DAH and at harvest. To avoid any edge effects plant samples were taken from the middle rows from each container and time point as well as a 15 cm deep soil sample. After the collection each sampled plant was further divided into three primary plant organs: Spike, stem (incl. leaf) and roots. A total of 576 individual samples were recorded for further analysis.

In experiment 2, N was applied 21 days after sowing (0 DAT), when the majority of the plants reached the third to fourth leaf stage (Zadoks 13–14). Each contaniner received 1 g N in the form of 5 atom % excess $K^{15}NO_3^-$. Samples were taken five times at 0 DAT, 14 DAT, 28 DAT, 42 DAT and at harvest by collecting one representative plant per container and time point as well as a 10 cm deep soil sample. In experiment 2, 180 individual samples were collected for further analysis but no separation of the collected material was conducted as the sample collection was done at earlier developmental stages.

Sample preparation and analysis

A sub-sample was taken from each sample, and transferred into 10-ml bottles along with five stainless-steal beads per bottle, to grind each sample to a fine homogenous powder using a TissueLyser II® (Qiagen, Venlo, Netherlands). Afterwards, the samples were dried at 105 °C for three hours. For the isotope-ratio mass spectrometry, 10 mg from each plant sample and 25 mg from each soil sample were weighed into tin capsules in two replications, respectively. Sample combustion was performed using a Vario PyroCube (Elementar, Langenselbold, Germany). Here, the sample was combusted at 920 °C and N was oxidized to nitrogen oxide using injected oxygen gas in abundance and helium as carrier gas. After reduction on elemental copper, the N-fraction was injected into the Isotope-ratio mass spectrometer (IRMS) using an Isoprime®-IRMS (IRMS; Elementar UK, Stockport, UK). In the IRMS, sample peaks and the appropriate reference gas peaks were ionized and the ion ratio was quantified, using a 29/28-ratio for N. Further, the reference gases were calibrated against standard samples with a known isotope-amount ratio obtained from the International Atomic Energy Agency (IAEA, Vienna, Austria). Calculations were made using the Ion Vantage® software (Elementar UK, Stockport, UK).

Methods of calculation

N derived from fertilizer (Ndff) was calculated using Eq. 1 [28]. In this study all ^{15}N values were expressed in the atom percent excess, applying a correctional factor for background abundance (0.366%).

Equation 1:

$$Ndff(\%) = (B - A)/(C - A) * 100$$

where A is the ^{15}N abundance of the soil at the particular measurement, B is the ^{15}N atom percent excess in the plant material and C is the ^{15}N atom percent excess in the applied N fertilizer.

Calculation of NUE

In both experiments, NUE parameters were calculated at harvest according to Good et al. (2004). NUE of the grain weight (NUE_{GW}) was calculated for experiment 1 and represents the quotient of the grain weight harvested per container divided by the N supplied per container. In both experiments, NUE for the straw weight (NUE_{SW}) was calculated and represents the quotient of the straw weight harvested per container divided by the N supplied per container.

Assessment of growth parameters

In experiment 1, growth parameters were recorded weekly starting with tillering (Zadoks 21) using the PlantEye F500® (Phenospex, Heerlen, Netherlands). The PlantEye F500® is a multi-spectral 3D laser scanner able to detect a wide range of relevant growth parameters and growth indices, such as digital biomass, digital height, normalized differential vegetation index (NDVI), normalized pigment chlorophyll ratio index and plant senescence reflectance index (Fig. 2).

To gain a detailed view of the physiological properties, leaf dimensions were recorded every week in experiment 2, beginning 14 days after sowing (-7 DAT). From each container, four plants were selected randomly and used throughout the whole experiment to measure the leaf characteristics by measuring length and width as well as chlorophyll content index (CCI) and leaf temperature of the youngest fully elongated leaf and the second youngest fully elongated leaf according to [33]. CCI was measured using the CCM-200 Chlorophyll Content Meter© (Opti-Sciences Inc, New Hampshire, USA). The CCM-200 provides a rapid, non-destructive method for assessing chlorophyll content in leaves by applying the principles of optical absorption. The instrument uses two specific wavelengths, 653 nm in the red spectrum and 931 nm in the near-infrared spectrum. The measuring area of the device is 71 mm^2 with a diameter of 9.52 mm. To obtain a representative value for each measurement, two technical repetitions were conducted per leaf, which were then averaged. Leaf temperature was recorded by measuring the temperature of the bottom side of the leaf using an IR 260-8S Voltcraft® infrared thermometer (Conrad Electronic, Hirschau, Germany). In both experiments, the Zadoks Growth Scale was used as the guiding scaling [34].

Collection of yield and biomass data

In experiment 1, the aboveground biomass of each container was harvested at the ripening stage (Zadoks 91–92) and subsequently divided into two primary plant organs: spike and stem (incl. leaf). For all

Fig. 2 Customized version of the PlantEye® 3D-Scanner used in the DroughtSpotterXXL phenotyping platform

collected plant biomass samples, both, fresh and dry weight was recorded. Further, primary yield components such as grain yield per container, thousand kernel weight (TKW) as well and seeds per spike were analyzed. In experiment 2, both, the fresh and dry weight of the aboveground biomass of each container was recorded at the end of tillering (Zadoks 29). The remaining root material was extracted from the soil separately. Fresh and dry weight was determined for all collected plant biomass samples.

Statistical analysis

For traits that were recorded at harvest, such as grain yield, thousand kernel weight and shoot dry mass, analysis of variance was conducted using Eq. 2. For all traits for which Eq. 2 was used, an alpha of 5% was used as the significance level. An analysis of variance was conducted using Eq. 3 for traits that were recorded over several time points during the experiment. An alpha of 5% was used as the significance level for all traits, except for daily and cumulative transpiration in experiment 2, where an alpha of 10% was used as the significance level. All comparisons reported to show significant differences were tested at a significance level of alpha 5%. For the calculation of the analysis of variance, the R-language based package lmerTest was used (http://CRAN.R-project.org/package=lmerTest). Based on the analysis of variance least significant differences were calculated using the R-language based package agricolae (http://CRAN.R-project.org/package=agricolae).

Equation 2:

$$P_{ijlmn} = \mu + g_i + t_j + W_l + C_m + R_n + e_{ijlmn}$$

where P_{ijlmn} is the phenotypic value of the i^{th} genotype, in the j^{th} irrigation treatment, the l^{th} replication, the m^{th} column and the n^{th} row. μ is the overall mean, g_i describes the fixed effect of the i^{th} genotype, and t_j is the fixed j^{th} irrigation effect. W_l is the random effect of the l^{th} replication, C_m represents the random effect of the m^{th} column and R_n represents the random effect of the n^{th} row. The error term is represented by e_{ijlmn}.

Equation 3:

$$P_{ijklmn} = \mu + g_i + t_j + s_k + W_l + C_m + R_n + e_{ijklmn}$$

where P_{ijlmn} is the phenotypic value of the i^{th} genotype, in the j^{th} irrigation treatment, the k^{th} time point of measurement, in the l^{th} replication, the m^{th} column and the nth row. μ stands for the overall mean, g_i describes the fixed effects of the i^{th} genotype, t_j stands for the j^{th} irrigation treatment and s_k describes the k^{th} time point of measurement. W_l is the random effect of the l^{th} replication, C_m represents the random effect of the m^{th} column and R_n represents the random effect of the n^{th} row. The error term is represented by e_{ijlmn}.

Results

Phenotypic characterization of drought response

Yield and biomass data

Ning0604 completed its life cycle after 177 days from sowing until harvest while recipients required 232 days. From sowing, Ning0604 reached the heading date (HD; Zadoks 59) within 170 days, followed by Elixer and

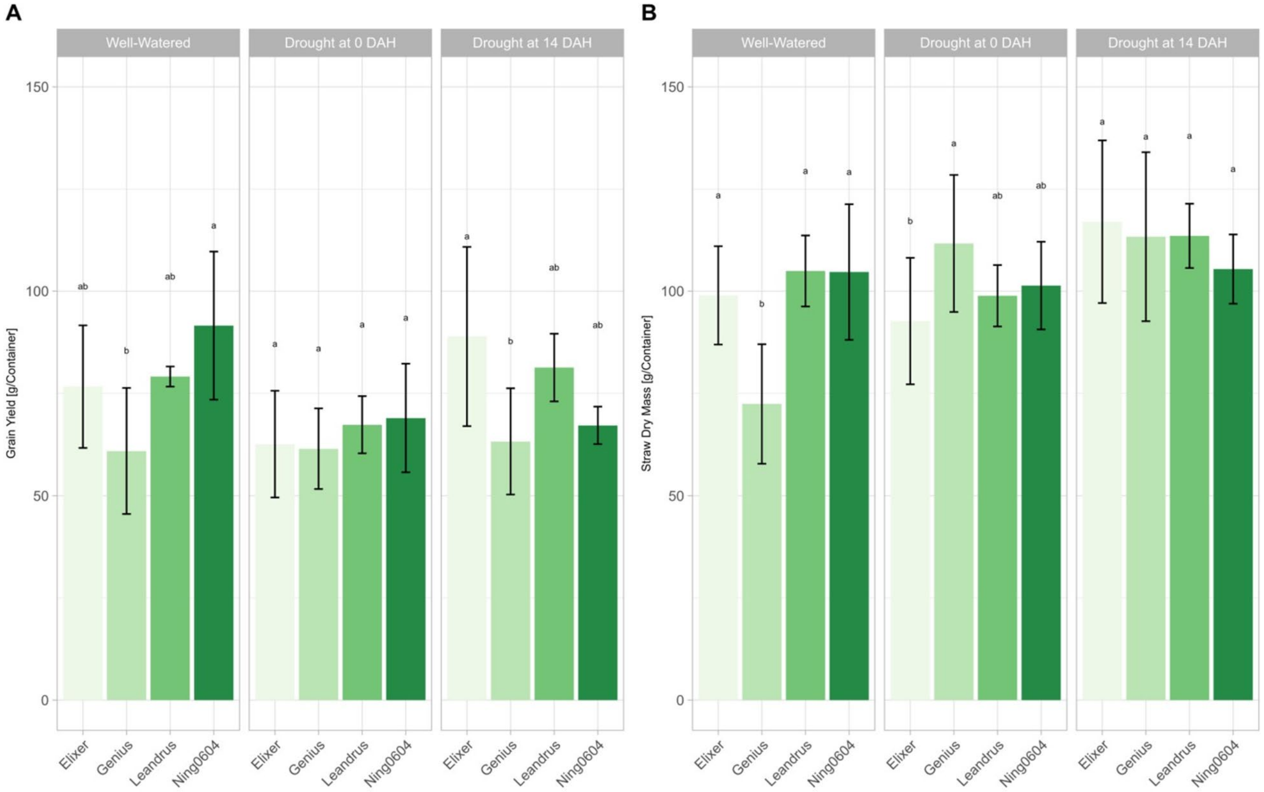

Fig. 3 Yield and straw dry mass data of experiment 1 (**A**) Grain Yield [g/Container] under contrasting irrigation treatments (**B**) Straw dry mass [g/Container] under contrasting irrigation treatments; colours stand for different genotypes; different letters indicate significance ($p < 0.05$) between the mean values of the genotypes in the specific irrigation treatment according to the Tukey test. Error bars represent standard errors

Genius, which took 195 days to develop fully emerged spikes. With 202 days, Leandrus needed the longest time to reach the heading date. Descriptive statistical parameters (minimum, maximum and arithmetic mean), variation (Var), standard deviation (SD) and coefficient of variation (CoV) for grain yield (GY), TKW, above ground dry mass and the dry mass values of the individual plant organs spike, straw and root (RDM) of experiment 1 are given in Additional file 2. The highest mean GY per container was observed for Ning0604 in well-watered treatment, which reached 91.57 g/container (Fig. 3A, Additional file 2). Further, significant genotypic differences can be observed in well-watered conditions and drought scenario 2. In all three treatment levels, Genius had the lowest GY, which was significantly lower under well-watered conditions compared to RDM donor and significantly lower than the GY of Elixer in drought scenario 2 (Fig. 3A, Additional file 2). Straw weight showed noticeably higher average values in drought scenario 2, compared to well-watered conditions, however without showing any significant differences between the genotypes. Significant differences were observed in the well-watered variant, where Genius had a significantly lower straw dry mass than the remaining three lines. Furthermore, Genius had a significantly higher straw dry mass than Elixer in drought scenario 1 (Fig. 3B, Additional file 2). For TKW, significant differences were observed in Elixer, which had the highest overall TKW in drought scenario 2 (4.01 g) and the lowest TKW in drought scenario 1 (3.38 g). The recorded biomass data revealed significant differences only for spike weight and RDM. For spike weight, the highest value can be found for Ning0604 in well-watered conditions with 125.90 g. For RDM, the highest value was found for Elixer in drought scenario 2 with 192.33 g (Additional file 2).

Descriptive statistics for experiment 2 can be found in Additional file 3. No significant differences were detected for dry mass, but as expected higher dry mass values were seen for all genotypes in the well-watered treatment. For RDM no significant differences between genotypes or treatment were observed. Furthermore, for all recorded traits, no significant differences were found between the recipient and the NILs of the respective genotype. For fresh matter content, the highest values were achieved by Genius NIL under well-watered conditions, for dry mass

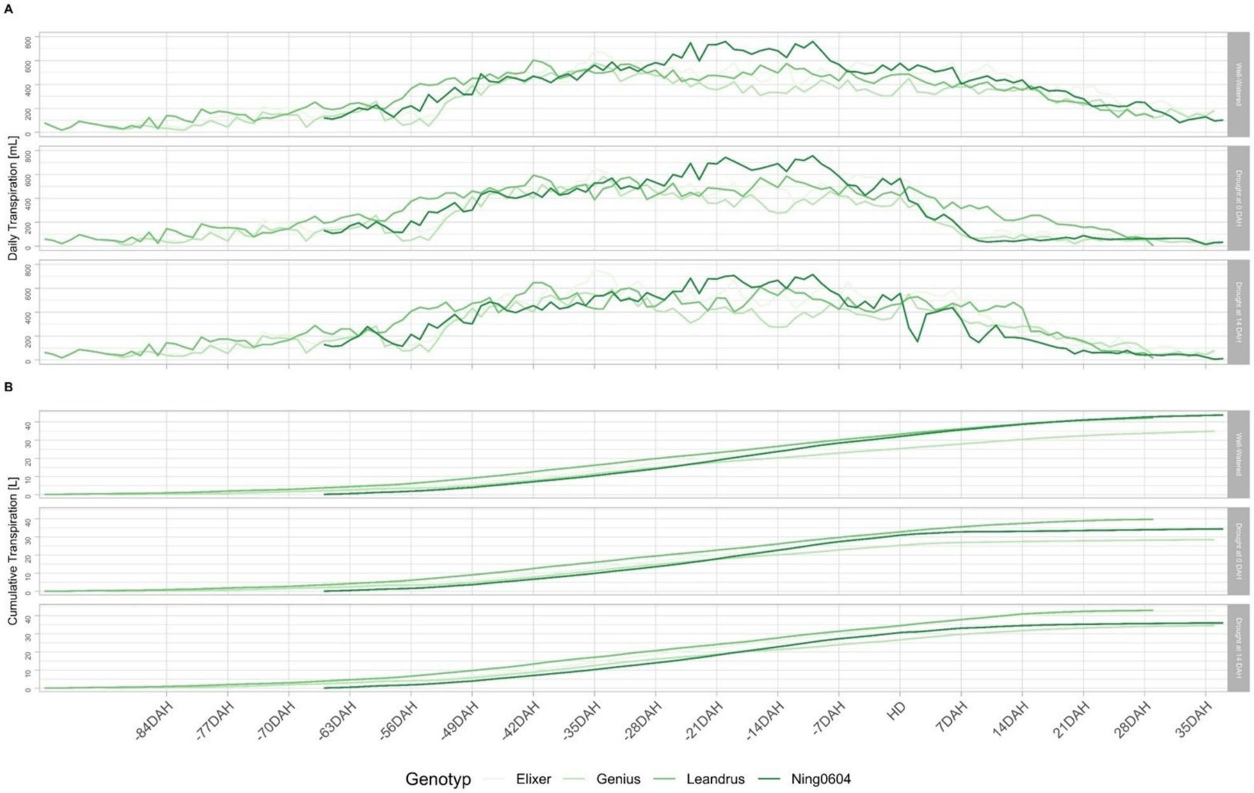

Fig. 4 (**A**) Daily transpiration [mL] and (**B**) Cumulative transpiration [L] under contrasting irrigation treatments in experiment 1. Time line is normalized around begin heading date (0 DAH) which corresponds as treatment begin; colours stand for different genotypes

the highest value was recorded for Elixer PAR in well-watered treatment (Additional file 3).

Above ground growth parameters

In experiment 1, the weekly PlantEye measurements of growth parameters digital biomass, digital height and NDVI revealed significant differences for digital biomass in Ning0604 compared to the three elite varieties across all treatments, beginning from the first measurement at -14 DAH until harvest (Additional file 4). A similar pattern was observed for digital height, where Ning0604 showed significantly higher plant height across all the treatments than the three recipients (Additional file 4). NDVI in experiment 1 revealed a distinct difference in the growth behaviour of Ning0604 compared to the recipients. Under well-watered conditions in drought scenario 2, Ning0604 showed significantly higher NDVI values than the recipients until 14 DAH, but its NDVI declined more strongly from 14 DAH until harvest. Interestingly, in drought scenario 1 this behaviour was not observed (Additional file 4). Leaf area, CCI and leaf temperature measurements in experiment 2 showed no significant differences (Additional file 5).

Analysis of gravimetrical data on water use parameters

To determine the impact of an increased root system on water use, we used a gravimetric approach in both experiments to measure daily and cumulative transpiration as well as WUE. In experiment 1, daily and cumulative transpiration were recorded from the beginning of tillering (Zadoks 21) until harvest (Zadoks 92). In experiment 2, the three recipients as well as their respective NILs were tested under the same conditions to measure the isolated effect of the root-associated haplotype blocks on daily and cumulative transpiration as well as WUE. Daily and cumulative transpiration were recorded throughout the entire duration of the experiment.

Cumulative and daily transpiration

Highly significant ($p < 0.001$) differences can be observed for daily transpiration within treatment levels as well as across treatment levels. The highest daily transpiration rates for these two treatments were reached by Ning0604, which transpired 734 ml under well-watered conditions and 689 ml in drought scenario 1. For drought scenario 2, the highest transpiration rate (749 ml) was recorded for Elixer (Fig. 4). Between 84 and 49 DAH, significant differences in daily transpiration were observed between

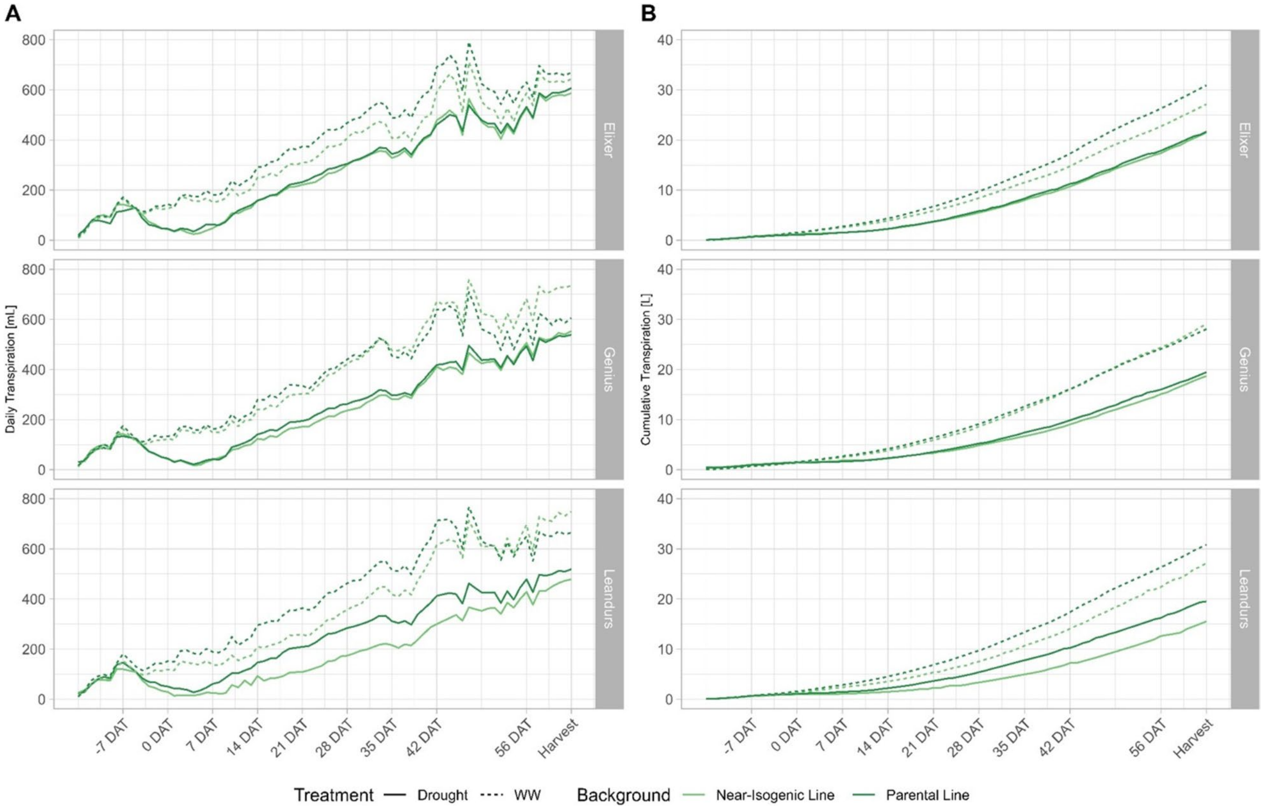

Fig. 5 (**A**) Daily transpiration [mL] and (**B**) Cumulative transpiration [L] of the different genotypes in experiment 2. Time line is normalized for the treatment begin (0 DAT). Different line types represent the genetic background; different colours stand for the different irrigation treatment

the genotypes in all three treatments. However, none of the genotypes tended to have a higher daily transpiration rate over the full course of two weeks (Fig. 4, Additional file 6). From -28 DAH until -14 DAH the Ning0604 showed significantly higher daily transpiration rates than the recipients in well-watered and drought scenario 1. A significantly higher daily transpiration rate for Ning0604 was observed from -7 DAH until HD in well-watered conditions as well as in drought scenario 1 (Fig. 4A, Additional file 6). The highest overall cumulative transpiration rate (42 L) was achieved by Elixer, Leandrus and Ning0604 in well-watered conditions, and by Elixer and Leandrus in drought scenario 2. The lowest cumulative transpiration under well-watered conditions was achieved by Genius, which had significantly lower transpiration compared to the other three genotypes from -21 DAH until harvest. In line with these findings, also in experiment 2 Ning0604 (35 L) and Genius (34 L) showed the lowest cumulative transpiration ($p < 0.05$). Further, significant differences in cumulative transpiration were observed from -84 DAH until -35 DAH. However, Leandrus had either the highest values or was at least in the same significance group as the genotype with the highest cumulative transpiration on the respective measurement day across all three treatments (Fig. 4B, Additional file 6). Except for the low transpiration of Genius under well-watered conditions, no significant differences were observed between -21 DAH and 14 DAH (Fig. 4B, Additional file 6).

Throughout experiment 2, significant ($p < 0.1$) differences were detected in daily and cumulative transpiration for Elixer (Fig. 5). Under drought conditions Elixer PAR showed a higher cumulative transpiration from 21 DAT until harvest than Elixer NIL. Furthermore, observation revealed lower transpiration of Leandrus NIL than Leandrus PAR under well-watered and drought conditions (Fig. 5B, Additional file 5). Similar to the cumulative transpiration, significant ($p < 0.1$) differences were found for the daily transpiration of Elixer under well-watered conditions. Here, Elixer NIL had lower daily transpiration between 0 and 21 DAT (Fig. 5A). Leandrus NIL recorded the highest daily transpiration rate under well-watered conditions (748.38 ml). A similar pattern was seen for the cumulative transpiration, which showed a continual gradient without reaching a plateau at any time. with the highest total value achieved by Leandrus PAR under well-watered conditions.

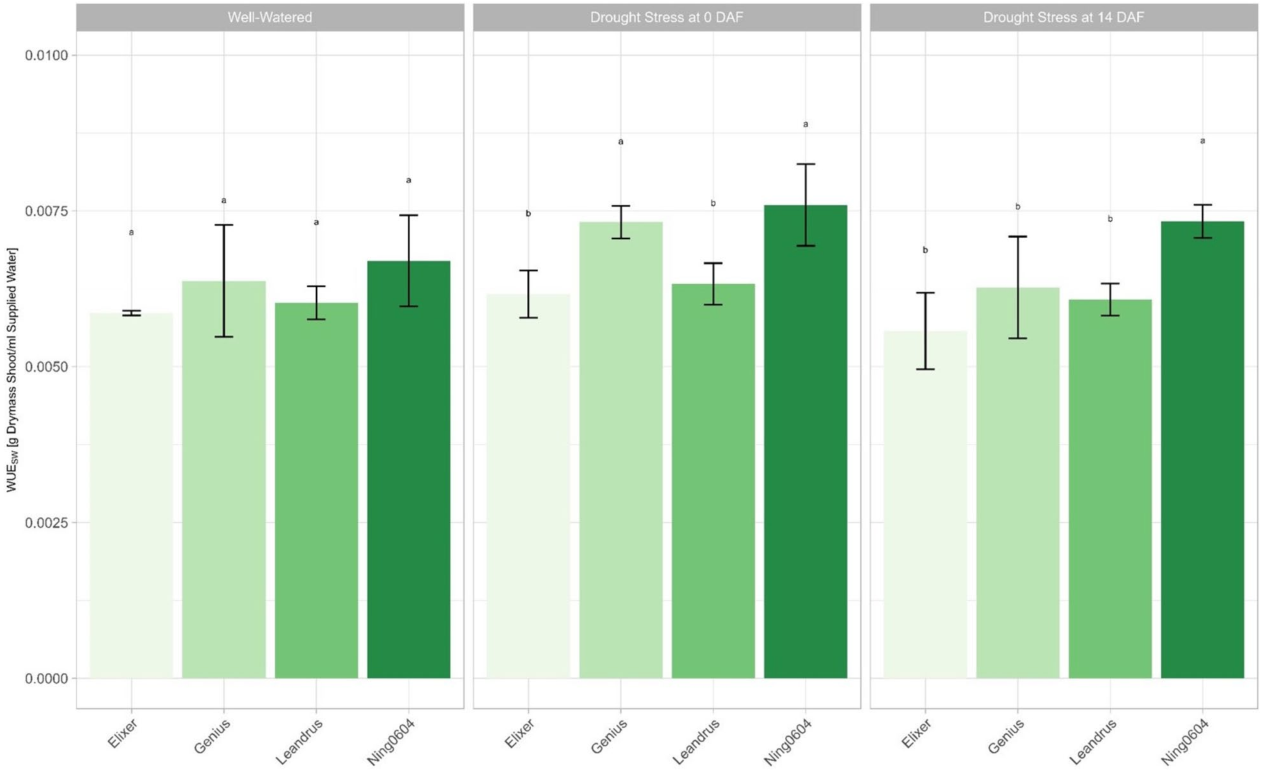

Fig. 6 Water use efficiency [g dry mass shoot/ ml supplied water] of shoot dry mass in experiment 1 under contrasting irrigation treatments Colours stand for different genotypes; different letters indicate significance ($p < 0.05$) between the mean values of the genotypes in the specific irrigation treatment according to the Tukey test. Error bars represent standard errors

Water use efficiency

Ning0604 achieved the highest WUE in all three treatments in experiment 1. In drought scenario 1, Ning0604 had the highest WUE of 7.5 mg dry mass shoot/ml of water supplied and thus had a significantly higher WUE than Elixer and Leandrus. Genius achieved a WUE of 7.3 g dry mass shoot/ml of water supplied in drought scenario 2 and thus was at the same significance level as Ning0604 (Fig. 6). Also, under drought scenario 2, Ning0604 had the highest WUE with 7.3 mg dry mass shoot/ml water supplied and thus had a significantly higher WUE than the three German elite varieties (Fig. 6). In comparison to drought scenarios 1 and 2, no significant differences could be observed in WUE under well-watered conditions. Here, Ning0604 achieved a WUE of 6.6 mg dry mass shoot/ml supplied water (Fig. 6). The WUE measured in experiment 2 showed no significant differences between the parental lines and the NILs in either treatment. However, genotype-treatment interaction for Elixer and Genius was observed (Additional file 7).

Phenotypic characterization of N uptake and N use

Nitrogen derived from fertilizer

In both experiments, 5 Atom % $K^{15}NO_3^-$ was applied to calculate Ndff in order to quantify the amount of N taken up and translocation from the timepoint of application (heading date). In both experiments, Ndff showed significant differences between and within the treatments. Furthermore, significant differences were observed in Experiment 1 between the different sampling time points and between the investigated plant organs. In all three treatments, Ning0604 showed the highest Ndff values in roots at 14 DAH. Especially in well-watered conditions and in drought scenario 2, Ning0604 also showed significantly higher N uptake (30%) compared to the three recipients, which did not exceed 20% at this time point. In drought scenario 1, the Ning0604 and Leandrus had significantly higher values compared to Elixer and Genius. This is particularly noteworthy since Leandrus is the only genotype of the three elite varieties that carries one of the two root-associated haplotype blocks (Fig. 7). In all three treatments all genotypes showed a decrease in straw Ndff from 14 DAH until harvest. As for 14 DAH in roots, the Ning0604 showed constant Ndff values of 15–17% in all three treatments, indicating a lower interaction between N-translocation and water availability. This tendency was also observed in the straw at 21 DAH, where Ning0604 showed constant Ndff values of 8–10%, compared to a decrease in the three elite lines. The straw

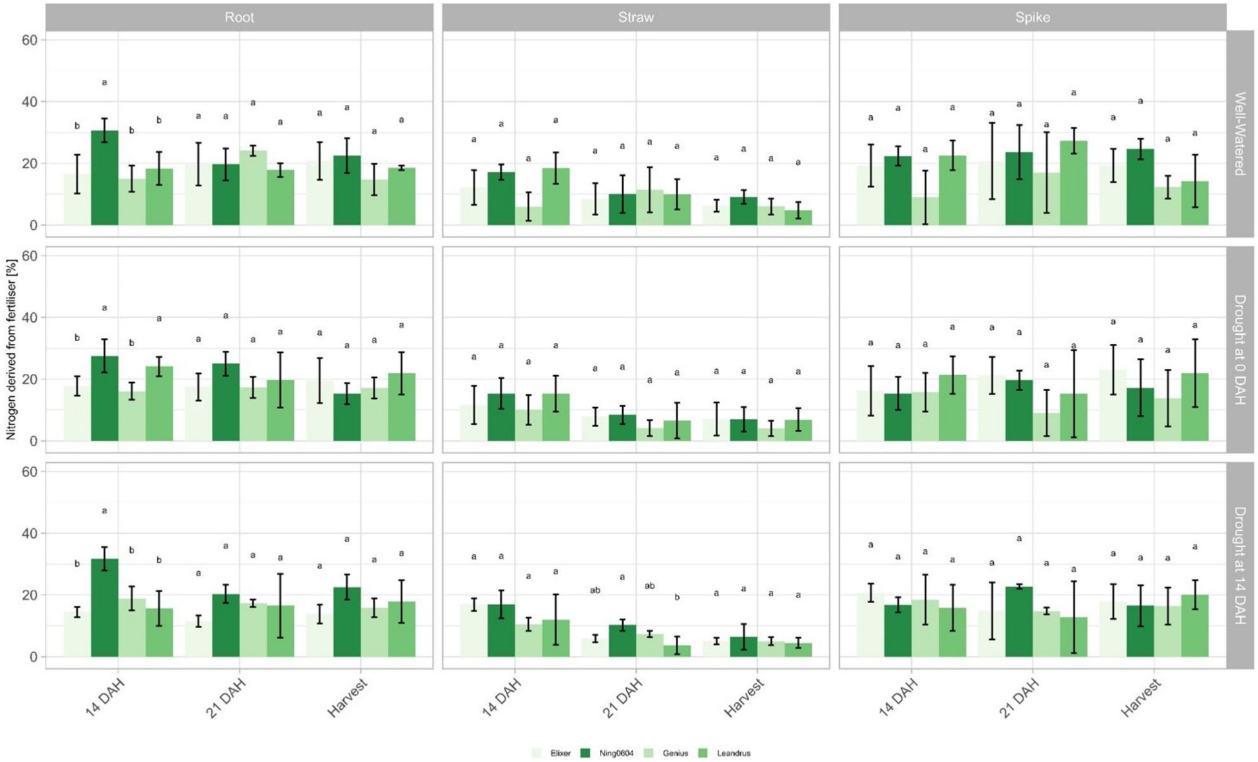

Fig. 7 Nitrogen derived from fertiliser in different plant organs and under contrasting irrigation treatments in experiment 1. Colours stand for different genotypes; different letters indicate significance ($p < 0.05$) between the mean values of the genotypes in the specific irrigation treatment and plant organ according to the Tukey test. Error bars represent standard errors

Ndff at 21 DAH in drought scenario 2 was particularly noteworthy, with Ning0604 showing significantly higher values (Fig. 7). For Ndff in spikes, no significant genetic differences were observed in any treatment or timepoint (Fig. 7).

Under well-watered conditions, comparable results were observed for Ndff in roots in experiment 1 at 14 DAH and 14 DAT in experiment 2. For two of the three elite varieties, the NIL showed significantly higher Ndff values than its respective recipient. In particular, the Ndff of Leandrus NIL reached only 12%, whereas the Leandrus NIL containing the Ning0604 Donor QTL exceeded Ndff values of 50% (Fig. 8).

Nitrogen use efficiency

The overall capacity of genotypes to translocate N was determined as the NUE_{GW} and NUE_{SW} in experiment 1 and as NUE_{SW} in experiment 2, using calculations according to Good et al. (2004). In experiment 1, NUE_{GW} revealed a significantly higher NUE of Ning0604 under well-watered conditions and a significantly higher NUE for Elixer under drought scenario 2. In drought scenario 1, no significant difference was found between the genotypes. Here, Ning0604 reached the highest NUE (Additional file 8A). Furthermore, genotype-by-treatment interactions were observed. For example, Leandrus NIL showed the highest NUE under well-watered conditions but not under drought stress.

NUE_{SW} revealed a significantly higher NUE under well-watered conditions in Elixer and Ning0604 compared to Genius, which exhibited the lowest NUE under well-watered conditions (Additional file 8B). No significant differences were found between the recipients and their respective NIL for NUE_{SW}. However, in well-watered conditions, the genotypes showed higher variation in NUE than in drought scenario 1, where NUE was similar in all genotypes between the NILs and their respective recipients. Furthermore, genotype-treatment interactions were identified, with Leandrus NIL showing an increased NUE under well-watered conditions while its recipient in both treatments showed constantly lower NUE. In comparison to Leandrus, an inverse behavior was seen in Genius, wherein the trend, but not significantly a higher NUE of the recipient was observed under well-watered conditions (Additional file 9).

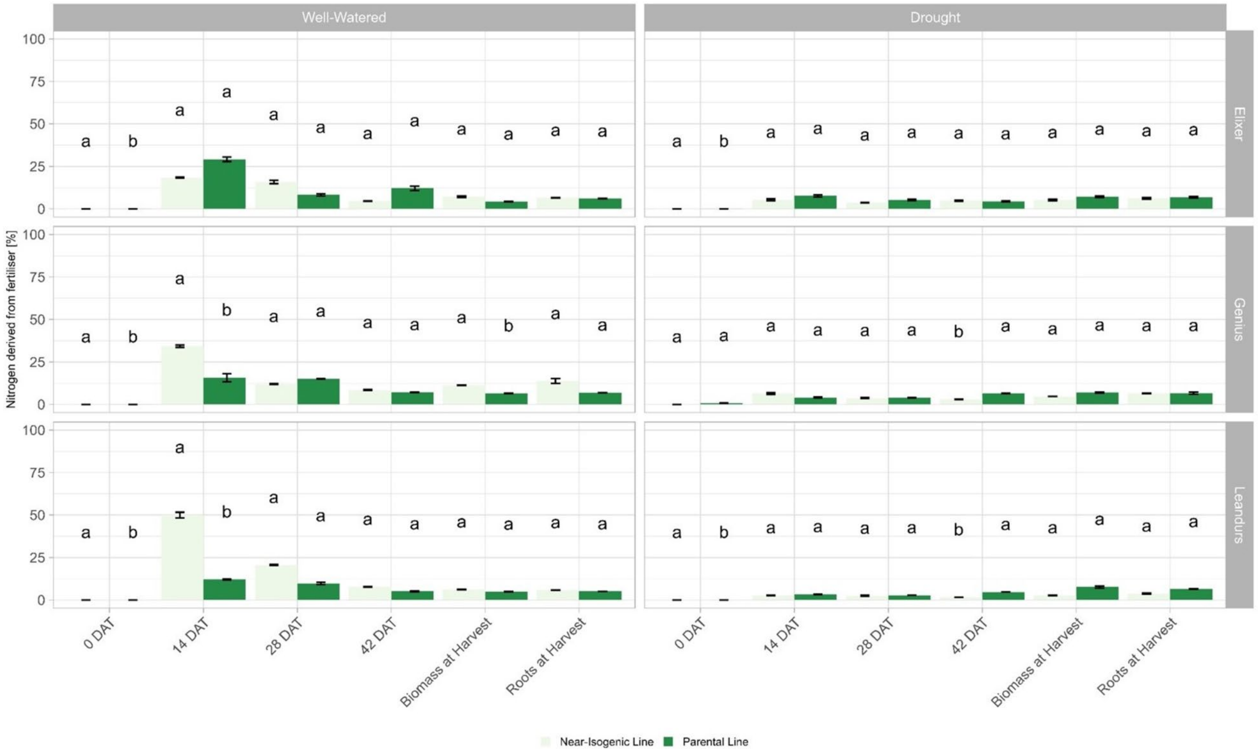

Fig. 8 Nitrogen derived from fertiliser in different plant organs and under contrasting irrigation treatments in experiment 2. Colours represent the genetic background; different letters indicate significance ($p < 0.05$) between the mean values of the genotypes in the specific irrigation treatment according to the Tukey test. Error bars represent standard errors

Discussion

QTL x background interaction potentially affects water-uptake

Several studies have reported improvements in water uptake ability and water use efficiency, as well as yield benefits, concerning root characteristics [2, 35, 36]. It was reported that the root angle and the number of seminal roots were found to be good indicators to select root systems that show good adaptive properties to drought stress conditions and thus can have a positive impact on yield performance [37]. A well-known example of a gene influencing root growth which also provides yield benefits under water-limiting conditions is *DeepRooting1*, described in rice [38]. The importance of root systems specifically adapted to certain environmental conditions such as soil texture and water availability has been frequently described [23, 24, 39, 40]. For example, it was reported that a 34% increase in grain size can be expected by using deep-rooted genotypes under limited water availability [23]. In the present study, we hypothesized that haplotype blocks associated with enhanced root biomass have a beneficial effect on WUE under different irrigation regimes. By marker-assisted backcrossing the root-associated haplotype-blocks were introgressed into three elite winter wheat backgrounds to compare NILs with their respective recipient lines and QTL donors under controlled drought-stress scenarios. One of three NILs carrying *Hap-5B-RDMa* and *Hap-5B-RDMb* showed lower cumulative transpiration than its respective parent under well-watered conditions. The fact this phenomenon was only observed in one background indicates an interaction between the genomic background provided by the parental line and the root-associated haplotype-blocks is present. Such QTL x background interaction was also observed in similar experiments, where certain NILs showed significantly higher grain yield than their parental line in both control and stress treatments, while other NILs showed lower grain yields in both treatments [41]. Multiple studies have reported the negative pleiotropic effect of introgressed genes on yield performance across changing environments [41, 42]. Under drought conditions, no differences between NILs and their donors had been observed for cumulative transpiration, daily transpiration or shoot WUE, which excludes the negative effects of the introgressed haplotype blocks on transpiration performance or WUE. The three NILs all showed equivalent RDM values to their common QTL donor in all treatments of both experiments. Although no conclusions can be drawn regarding water uptake based on RDM alone, our results indicate that *Hap-5B-RDMa* and *Hap-5B-RDMb* can increase WUE in a genotype-dependent manner. Thus, this study suggests closer investigations for root morphology or root growth behaviour and their impact on WUE, since the DroughtSpotter phenotyping platform is limited for the investigation of such traits. Further, since this study focused on phenotypic response on WUE, the insights in this study are exclusively drawn on morpholocial an physiological characteristics at the plant level. Several molecular studies have evaluated the regulation of root associated genes and their regulation under drought conditions in wheat [43–45] and barley [46]. Although the crucial role of molecular studies in understanding the underlying mechanisms is recognized, it is worth noting the limited molecular information on the influence of *Hap-5B-RDMa* and *Hap-5B-RDMb* on the regulation of drought tolerance genes. In this context it would be of particular interest to investigate the influence of *Hap-5B-RDMa* and *Hap-5B-RDMb* on the enzymatic activity on possible effects of antioxidant activity on drought stress tolerance [47–49].

Root associated haploblock-types affect Nitrogen derived from fertilizer

The second objective of this study was to investigate the effect of *Hap-5B-RDMa* and *Hap-5B-RDMb* on NUE in general and on NupE and NutE in particular. Since the root is the primary organ for nutrient uptake, several studies have investigated the effect of root-associated traits on NUE [50–54]. Accordingly, it is well known that the N accumulated before anthesis is the main source of grain N. Hence, genetic variation in grain yield and grain N content is mainly explained by pre-anthesis N accumulation rather than post-anthesis N remobilization [22]. Increased Ndff may indicate an increased NupE before anthesis, and corresponding to this assumption we found that NILs carrying *Hap-5B-RDMa* and *Hap-5B-RDMb* exhibited higher Ndff than their respective recipients under well-watered conditions. In both hexaploid and tetraploid wheat, diverse studies have addressed the question of whether NupE or NutE contributes more strongly to improving NUE and described genetic variation for NupE and NutE [22, 55–59]. In a two-year experiment using three N levels, it was reported that 62–70% of the genetic variation for NUE was explained by NupE [58]. Comparable results were reported by other studies, where 54–63% of NUE was explained by NupE [55, 57]. The lack of significant genetic-environmental interactions in this study may be put into perspective by other, more recent studies, which found that the influence of NupE and NutE on NUE depended on soil N availability [59]. Under low and high N availability, NupE and NUE correlated significantly with each other, while NutE showed significant effects on NUE only under high N availability [57]. Since this causes NupE to exert a strong influence on NUE at several N levels, a higher importance was assigned to NupE [59]. In contrast, other studies found interactions in NUE between different N levels, but a higher significance of NutE [58]. This is consistent with studies, which reported a variation of NUE under low-N conditions being largely described by NutE [22]. Similar results were reported for maize, where the genetic variation of NUE under high-N conditions was mainly described by NupE, whereas NUE variation was mainly described by NutE under low-N conditions [60]. In both of our experiments, positive effects on Ndff were detected by genotypes carrying the haplotype blocks *Hap-5B-RDMa* and *Hap-5B-RDMb*. Furthermore, the results indicate that *these haplotype blocks* are particularly beneficial under well-watered conditions. In addition, the donor lines also show increased Ndff values in drought scenario 2, where sufficient water was presumably available at 14 DAH (Fig. 7). Two out of three NILs (Genius and Leandrus) also showed higher Ndff than their respective recipient lines under well-watered conditions. Thus, as with the previously described relationship between *Hap-5B-RDMa* and *Hap-5B-RDMb* and the transpiration parameters, QTL interactions with the genetic background of the recipient lines were also observed for the well-watered variant in combination with Ndff as the NIL of Elixer shows lower Ndff than its parental line (Fig. 8).

Hap-5B-RDMa and Hap-5B-RDMb have no pleiotropic effect on pre-anthesis growth parameters

The third aim of this study was to test whether *Hap-5B-RDMa* and *Hap-5B-RDMb* have an impact on above ground growth characteristics Several previous studies have shown significant effects of QTL for root architecture and root growth on aboveground growth behaviour [37, 59]. In contrast, no effects of *Hap-5B-RDMa* and *Hap-5B-RDMb* on aboveground biomass were detected. This is in line with similar studies that also failed to detect significant correlations between root and shoot ratios [62]. The lack of influence on aboveground growth factors rules out the pleiotropic effects of *Hap-5B-RDMa* and *Hap-5B-RDMb* on shoot biomass. Also, drought-induced changes in root development or RDM that have been reported in other studies [20, 63] could not be confirmed. Several studies also reported positive effects of root traits on grain yield [61, 62, 64]. Unfortunately, the container size of the *DroughtSpotter L* platform used for experiment 2 is insufficient to obtain meaningful grain yield estimates, hence, we only investigated the vegetative growth phase in experiment 2, and at this stage are unable to conclude the potential effects of *Hap-5B-RDMa* and *Hap-5B-RDMb* on grain yield, or other traits determining agronomic performance in field conditions. Future experiments are required to enable more insight into these relationships.

Implications for further studies

The results presented here show that ^{15}N tracers, in combination with high-resolution phenotyping platforms, can be effectively exploited to study N uptake and N translocation under different, defined water availability conditions. In terms of N translocation, it is particularly important to consider changing relationships between sink and source during plant development. Thus, the N utilization must be considered in relation to the metabolization rate of the source organ [65]. Especially during post-anthesis, when the metabolization rates of the yield-determining organs increase, the leaf tissue serves as a valuable N source. In this study, this process was visible as a gradual reduction of Ndff in straw and leaf tissues, respectively, which are a major source of N during senescence. This finding is consistent with studies that described remobilization into the yield-producing organs as an important factor for high grain yields [66]. Various other studies also indicated the importance of high N remobilization for high grain protein content [67, 68].

Another advantage of the setup used in experiment 1 is that it allowed a detailed investigation of genotypic differences in N uptake after anthesis, which is reported to have a particularly high influence on the grain protein deficit [68–70]. One hypothesis concerning the physiological mechanisms underlying genotypic variability for this trait is that improved root penetration of the soil leads to improved N uptake [70]. Since Ning0604 and two out of three NILs showed higher Ndff, our study supports this hypothesis. This study extends insights into the impact of genetically altered root biomass on wheat NUE and WUE under different water availability scenarios. They also enabled the identification of relationships with other key traits, such as biomass. The use of ^{15}N tracers for tracking N uptake and remobilization in a large-container precision phenotyping system proved to be a proficient method to measure WUE, NUE and their interaction with above ground biomass with a high resolution under field-like growth conditions. In particular, the ability to assess the uptake of applied N under different irrigation treatments, concerning the impact of root traits on N translocation highlights the value of the experimental setup. Nevertheless, potential interactions due to environmental and developmental factors must be clarified for a better understanding, since factors such as the timing and rate of N application, type and induction of drought stress as well as crop management factors such as sowing may also impact NUE and WUE. This study conducted a full growth cycle trial only in experiment 1, and since no NILs were used in this particular trial the full understanding of the impact of *Hap-5B-RDMa* and *Hap-5B-RDMb* on yield performance is still limited. Hence, two approaches to validate the effect of *Hap-5B-RDMa* and *Hap-5B-RDMb* on yield performance were conducted. First, full growth-cycle experiments in high-resolution phenotyping platforms will provide more detailed information on N uptake and N translocation under different irrigation treatments and potentially help identify suitable target environments for genotypes with enhanced root biomass. Further, although the design of the DroughtSpotter system enables a simultaneous and continuous examination of makro physological traits, we recommend the additional examination of micro physiological traits, such as stomatal aperture, to obtain higher resolutions on trait characteristics and interactions. Secondly, field trials of the NILs and their respective recipients will enable a more detailed evaluation of their performance in diverse environments. Meanwhile, the initial results presented here suggest that introgression of QTL conferring altered root biomass can be a rapid, useful and valid approach to improve NUE in wheat breeding programs.

Conclusions

The manuscript presents a combination of unique, innovative gravimetric phenotyping platforms to assess transpiration throughout the growing season, and 15N tracers to study nitrogen uptake and translocation. It was shown that the previously identified QTL on chromosome 5B, which is associated with a larger root system, alters both transpiration and nitrogen uptake and is therefore a potential target for breeding more efficient varieties. However, there is also an interaction with the background of the respective genotypes and the specific drought scenario. Therefore, future studies should investigate the interaction of the locus with divergent environmental scenarios and potential epistatic effects in different genetic backgrounds.

Abbreviations

CCI	Chlorophyll content index
CoV	Coefficient of variation
DAH	Days after heading date
DAT	Days after treatment begin
DS L	DroughtSpotterL
DS XXL	DroughtSpotterXXL
GY	Grain yield
HD	Heading date
IRMS	Isotope-ratio mass spectrometer
Ndff	Nitrogen derived from fertilizer
NDVI	Normalized differential vegetation index
NIL	Near-Isogenic Line
NUE	Nitrogen use efficiency
NupE	Nitrogen uptake efficiency

NutE	Nitrogen utilization efficiency
PAR	Parental line/Recipient
QTL	Quantitative trait locus
RDM	Root dry mass
SD	Standard deviation
TKW	Thousand kernel weight
Var	Variation
WUE	Water use efficiency

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-04756-8.

Additional file 1. Mean temperature [°C] and mean relative humidity [%] for experiment 1 and experiment 2.

Additional file 2. Descriptive statistics for collected harvest data in experiment 1.

Additional file 3. Descriptive statistics for collected harvest data in experiment 2

Additional file 4. Digital growth parameters recorded in experiment 1 using the PlantEye F500® multi-spectral 3D laser scanner

Additional file 5. Descriptive statistics for transpiration data as well as physiological growth parameters recorded in experiment 2 from -7 DAT until harvest

Additional file 6. Descriptive statistics for cumulative transpiration [L] and daily transpiration [ml] recorded in experiment 1 from -84 DAH until 35 DAH

Additional file 7. Water use efficiency calculated for experiment 2.

Additional file 8. Nitrogen use efficiency of grain weight and shoot drymass under contrasting irrigation treatments in experiment 1.

Additional file 9. Nitrogen use efficiency of shoot dry mass under contrasting irrigation treatments in experiment 2.

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Authors' contributions

AS and RJS conceived the study. SV, AE and ALM performed the experiments. CB conducted the IRMS analysis. SV and AS analysed the phenotypic data. SV wrote the manuscript with further input from AE, ALM, CB, SR, RJS and AS. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The data supporting the findings of this study are available from the corresponding author, Stjepan Vukasovic (Stjepan.Vukasovic@agrar.uni-giessen.de), upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Publisher's Note

5. Discussion

5.1. Demand to evaluate plant adaptation traits to increase drought stress resistance in wheat and sorghum

Wheat and sorghum are important crops for ensuring human nutrition in many parts of the world due to their high calorie content and versatile processing options (Sharma et al., 2015). Wheat is the third most important crop globally in terms of annual production (FAOSTAT, 2022). It is projected that annual demand will reach approximately 900 million metric tonnes by 2050, based on assumptions about population growth, economic developments, and changes in dietary habits (Pingali, 2007; Gerbens-Leenes et al., 2010; Alexandratos and Bruinsma, 2012).

Long-term forecasts indicate that heat and drought stress scenarios will gain in frequency and intensity in Europe. This is particularly evident in Mediterranean regions such as France, Italy, Spain, and Greece. Several studies have highlighted this issue (Semenov, 2008; Trnka et al., 2014; Wilcox and Makowski, 2014; Forzieri et al., 2016; Lüttger and Feike, 2018; van der Velde et al., 2011; Fontana et al., 2015; Mavromatis, 2015; Páscoa et al., 2017). Globally, there is expected to be an increase in yield losses, particularly in significant parts of South America and Africa, due to a rise in drought stress scenarios (IPCC, 2022). The growing regions of south-western and north-eastern Australia are also expected to be affected by declining rainfall, with anticipated yield declines of 30-58% due to reduced rainfall (Farooq et al., 2014; Lobell et al., 2015). It can be assumed that the demand for sorghum in these growing regions will continue to increase in the future. Therefore, ensuring high productivity of these two crops in the future is of utmost importance.

5.1.1. Morphology and phenology of early vigour and its genetic components

Several studies have identified EV as a potential trait to increase drought stress resistance, particularly in the Mediterranean climate conditions of the north-eastern cropping areas of Australia (Nusinow et al., 2011; Boden et al., 2014; Moore and Rebetzke, 2015; Duan et al., 2016). Although many studies have already demonstrated the positive effect of EV, this trait has only been included as a trait of interest in commercial breeding programmes to a limited extent. The reason for this is that, on the one hand, there is insufficient information on the genetic variation of the trait and, on the other hand, the commercial value of the trait is still questionable (Rebetzke et al., 2017). To determine the effects of EV on yield, a better understanding of the morphological and phenological understanding of the physiological mechanisms of the trait and their genetic control is required. In addition, efficient and cost-effective phenotyping methods are necessary to evaluate EV in wheat. The results presented in chapter 2 provided insights in the characterisation of morphological and phenological and its genetical components that contribute to EV. Here a population of 685 spring wheat genotypes deriving from a multi-reference nested-association mapping (MR-NAM) were tested over three years under field conditions. Further, a subset of 210 genotypes was formed to grant a detailed evaluation of EV under controlled conditions.

Numerous studies have highlighted the significant influence of leaf width on a specific leaf area, subsequently impacting EV (Rebetzke and Richards, 1999; Richards and Lukacs, 2002; Maydup et al., 2012). The study shows a significant correlation between EV variation and the length of leaves in both field and greenhouse conditions. Our findings show a positive correlation between EV variation and the length of leaves in both field ($p < 0.05$; $r = 0.39$) and greenhouse conditions ($p < 0.05$; $r = 0.38$). The length of leaves is crucial in developing a larger leaf area, leading to increased EV. This observation is consistent with prior research that has shown a significant positive correlation between the size of the second leaf and its length (Nusinow et al., 2011; Boden et al., 2014; Moore and Rebetzke, 2015; Duan et al., 2016). Further, the data indicates that the second leaf develops more rapidly in the greenhouse compared to field studies, which was previously observed by Rebetzke et al. (2007). This suggests that EV can be measured at earlier growth stages under controlled conditions than in natural field settings. Previous research has shown that embryo size, a trait that is highly heritable, is closely associated with leaf area (López-Castañeda et al., 1996; Rebetzke and Richards, 1999; Aparicio et al., 2002; Moore and Rebetzke, 2015). Therefore, in our study, we used seed weight as an indirect measure to evaluate the impact of embryo size on EV, as embryo size increases with seed weight in both wheat and barley.

The greenhouse experiment aimed to establish a positive relationship between embryo size and EV using seed weight. However, no significant correlation was found with any of the measured leaf parameters. Previous research has indicated a beneficial effect of embryo size on EV in wheat (Rebetzke and Richards, 1999; Richards and Lukacs, 2002; Moore and Rebetzke, 2015). However, studies have shown that seed weight alone is not sufficient to fully account for the total variation in EV (Maydup et al., 2012). Instead, seed density has been proposed as a potentially more influential factor in determining EV (Ball et al., 2011). This may explain the lack of correlation between seed weight and EV parameters in our study. The biomass was recorded in the GH experiment, and a significant correlation was found with the area of both the first (L1) and second (L2) leaves. This suggests that genotypes with greater EV tend to have increased above-ground biomass. Richards et al. (2019) also noted that lines with enhanced above-ground biomass were strongly linked to higher grain yields.

Our recent phenotypic study provided deeper insights into the EV trait in wheat, elucidating its main characteristics and its correlation with biomass. We successfully developed effective methods to detect EV both in field conditions and controlled environments. Notably, the correlation between leaf ($p < 0.05$; $r = 0.39$) length parameters and NDVI underlines the significant potential of growth indices, especially with the advancements in drone-based phenotyping platforms as highlighted by Shi et al. (2016). However, it's crucial to acknowledge the potential influence of environmental factors, such as sowing time, depth, rate, and row spacing, on EV. These factors can complicate yield predictions based on EV, as the trait is observed at an early developmental stage and its relationship with yield can be influenced by various environmental stressors, particularly during key growth phases like tillering and flowering. Despite these challenges, the importance of EV for optimal crop establishment and its potential impact on early-stage yield cannot be overstated. We recommend incorporating EV measurements, using NDVI, in performance evaluations like stay-green trials for better understanding its role and value in targeted environments. Furthermore, our genomic analysis identified specific QTL associated with EV. These QTL are either co-located or closely linked to key genes that regulate plant development, including plant height, coleoptile length, stay-green, and vernalisation genes. This supports the hypothesis that EV is a trait controlled by pleiotropic genes. Given that EV is influenced by numerous QTL with minor effects, marker-assisted selection methods might be challenging. However, genomic prediction approaches offer a promising alternative for future breeding efforts, allowing for more precise and efficient selection of desirable traits.

5.1.2. Use of precision phenotyping methods to dissect physiological responses in sorghum on different drought stress scenarios

Sorghum is a multipurpose crop, since it is used for human nutrition, animal feed, bioethanol production as well as base material for bioplastic and bio-concrete products (Belton and Taylor, 2004; Mace et al., 2013; Windpassinger et al., 2015; Rhodes et al., 2017). However, in order for sorghum to perform optimally for the intended areas of use, a better understanding of drought adaptation methods is needed, as drought stress can reduce yields by 20 to 60% depending on intensity and incidence (Prom et al., 2014; Amelework et al., 2015; Perrier et al., 2017). The work presented in chapter 3 set out to finely characterize key agro-physiological responses and water use of one caudatum–kafir genotype, with superior drought tolerance (Windpassinger et al., 2015) subjected to different drought scenario. For this purpose, a greenhouse experiment was conducted as a full-growth cycle trial using a phenotyping platform designed for drought stress-related experiments using growth containers placed on weight scales, which record weight deviations every five minutes throughout the whole experiment (Stahl et al., 2020). In order to investigate in detail, the effect of different intensity and timing of drought on physiological traits, four different irrigations treatments were used. A well-watered (WW) treatment was drawn up as a control. Further three drought treatments were conducted, an early vegetative drought stress (DS1), an early vegetative drought stress including a recovery irrigation (DS1R) and a late vegetative drought stress (DS2).

The results show that the applied drought stress had significantly ($p < 0.05$) affected plant phenology and plant morphology. The plants in drought conditions reached flag leaf and flowering later than those grown under well-watered conditions. Further, plants in DS2 conditions rapidly lost most of their green leaves, which could be an adaptive strategy to limit water loss through transpiration. Previous studies have also shown that plant phenology, is strongly affected by drought stress (Craufurd et al., 1993). This influence on plant phenology has also been shown by Rakshit et al. (2017), who observed a genotype-dependent increase in days to anthesis under drought stress.

Drought stress affect plant morphology depending on growth stage. DS1 led to a 25% height reduction compared to well-watered plants, while DS2 caused a 35% height reduction. Leaf emergence showed different phases for drought-stressed plants and for well-watered ones. In DS1 and DS2 leaf production slowed down from 5 to 20 days, while recovering to a 5-day interval in DS1R. Early drought stress reduced plant height, leaf number, and size. Previous studies have also reported a decrease in plant height, above-ground biomass and grain production under early vegetative drought stress conditions (Akman et al., 2021; Gano et al., 2021; de Menezes et al., 2022).

The results indicated that drought stress had a more detrimental impact on all physiological parameters during the early vegetative stage compared to the late vegetative stage. Drought stress led to decreased transpiration and net photosynthesis rates, along with reduced stomatal conductance. This reduction in stomatal conductance likely resulted from the limited availability of CO_2 for photosynthesis due to stomatal closure. Numerous studies have reported that stomatal closure under drought stress inhibits plant photosynthesis to minimize water loss through transpiration (Tsuji et al., 2003; Fracasso et al., 2017; Wang et al., 2017). Conversely, an increase in intercellular CO_2 concentration was observed under drought conditions, indicating the presence of CO_2 in substomatal cavities. However, this CO_2 was not effectively transferred to the carboxylation sites in the chloroplasts, suggesting that the inhibition of photosynthetic activity was due to non-stomatal limitations, likely mesophyll conductance. Previous studies have similarly reported non-stomatal limitations of photosynthetic activity in sorghum, maize, and Medicago (Li et al., 2007; Hasan et al., 2017; Endris et al., 2021). Mesophyll conductance has been identified as a factor reducing carbon fixation in many crops (Ermakova et al., 2001; Olsovska et al., 2016; Sonawane et al., 2020). The Fv/Fm ratio has been widely used as an index to assess crop resistance to drought stress (Hasan et al., 2017). In this study, both early and late vegetative drought stress resulted in a reduction in the maximum yield of photosystem II (Fv/Fm). This decrease in Fv/Fm, observed in sorghum in previous studies (Zhang et al., 2019; Galicia-Juárez et al., 2021), could indicate damage to the photosystem II reaction center, as described in Chapter 3. Additionally, a 14% decrease in chlorophyll content was noted during early and late vegetative drought stress. This reduction in chlorophyll content suggests chlorophyll degradation due to chloroplast damage, thus limiting photosynthesis (Kapanigowda et al., 2013).

5.2. The root: Key plant organ to increase transpiration efficiency and NupE?

Chapter 1.5 emphasised the significance of N as a fertilizer in modern wheat production. Its quality and quantity make it the most important plant nutrient for farmers to achieve their economic goals (Payne et al., 1987; Würschum et al., 2016; Swarbeck et al., 2019). However, the use of nitrogen fertilization can also negatively influence ecosystems due to leaching or volatile atmospheric emissions. Thus, breeding varieties with higher NUE and WUE is becoming more important to maintain yield and quality parameters while reducing the environmental footprint (Lupini et al., 2020). Yet, despite sufficient information on the components of NUE, there is a need for a deeper understanding of the genetic factors, morphological and physiological traits that influence the components of NUE under specific environmental conditions and developmental stages (Gaju et al., 2011; Hawkesford et al., 2014).

As the primary interface for water and nitrogen uptake, improved root systems could increase NUE and WUE. Although many studies propose an increased and deeper root system, it is important to understand root related traits in the context of specific environment (Ober et al., 2021). Voss-Fels et al. (2018a) were able to demonstrate that root dry mass was lower in European material compared to Chinese landraces within a diversity panel under controlled growth conditions. This suggests that modern elite crop varieties may have undergone unintended negative selection against advantageous root traits due to the focus on aboveground traits in breeding programmes. This negative selection may have resulted in a reduction in aboveground biomass and yield (Voss-Fels et al., 2018a). Nevertheless, Voss-Fels et al. (2019) discovered that in a wheat set comprising regionally adapted lines, modern varieties demonstrated the highest yields under both high-input and low-input growing conditions. The pleiotropic effects of favourable aboveground traits may be one reason for this negative effect on root characteristics. For instance, several reduced height genes have been shown to have negative pleiotropic effects on root traits. These genes have played a significant role in improving the harvest index of modern elite varieties (Bai et al., 2013). However, roots are of particular significance as the primary organ for nutrient and water uptake in increasing NupE and NUE for intensive wheat production systems (El Hassouni et al., 2018; Voss-Fels et al., 2018; Alahmad et al., 2019). For instance, a root system with a higher root length density is considered to have a greater potential to increase NupE (Lammerts van Bueren and Struik, 2017). In particular, the mobilisation of N before anthesis contributes more to N storage in the grain than remobilisation after anthesis (Gaju et al., 2011). Early and extensive development of the root system in the initial growth stages can enhance pre-anthesis N uptake and, therefore, increase the total NUE (Gaju et al., 2014). Therefore, it is crucial to investigate the influence of an increased root system on the mobilisation of nitrogen (NupE) and its translocation (NutE) to obtain a comprehensive understanding of nitrogen use efficiency (NUE). The use of ^{15}N-labelled fertilisers is particularly helpful for tracking the utilisation of applied nitrogen from a defined developmental stage (Chen et al., 2016). Voss-Fels et al. (2018a) suggest that linkage drag can result in unfavourable root systems in modern elite cultivars. For instance, European elite wheat cultivars lack two haplotype block alleles that contain quantitative trait loci (QTL) with positive effects on root dry mass (RDM) due to linkage drag with a QTL controlling heading date. The study by Voss-Fels et al. (2018) presents two beneficial haplotype blocks, *Hap-5B-RDMa* and *Hap-5B-RDMb*, which are highly conserved and exclusively present in Chinese wheat varieties. Most modern wheat breeding programs do not select for genotypes with positive root traits due to the difficulty of phenotypic quantification (Wasson et al., 2014). Marker-assisted selection for root-associated QTL could contribute to overcome this bottleneck, provided that these QTL have been thoroughly tested and their positive effects validated.

Chapter 4 presented research on the impact of haploblock types Hap-5B-RDMa and *Hap-5B-RDMb* on water use, N uptake, and aboveground plant organs. The goal was to provide potential solutions for practical plant breeding by altering the root system in modern wheat lines. Two experiments were conducted using precision phenotyping platforms for controlled drought stress treatment. Experiment 1 compared four winter wheat genotypes, including the Chinese variety Ning0604, which carries an allele on chromosome 5B positively associated with higher root dry biomass, with three elite German varieties. Experiment 2 compared NIL of the three elite varieties, each containing introgressions of the QTL on chromosome 5B linked to root dry mass. In both experiments, nitrogen partitioning was monitored by isotope discrimination after fertilisation with 5 Atom % ^{15}N-labelled KNO_3. This thesis presents results indicating that haplotype blocks associated with enhanced root biomass have a beneficial effect on WUE under different irrigation regimes. One of the three NILs carrying *Hap-5B-RDMa* and *Hap-5B-RDMb* showed lower cumulative transpiration than its respective parent under well-watered conditions. The observation of this phenomenon in only one background suggests an interaction between the genomic background of the parental line and the root-associated haplotype-blocks. Similar experiments have also shown a QTL x background interaction, where certain NILs exhibited significantly higher grain yield than their parental line in both control and stress treatments, while others showed lower grain yields in both treatments (Blanco et al., 2006). Several studies have reported the negative pleiotropic effect of introgressed genes on yield performance across changing environments (Blanco et al., 2006; Jones et al., 2020). The work presented in chapter 4 cannot confirm this observation, since no yield was recorded for the NILs and their donors. However, regarding the recorded above ground traits no difference between the NILs and their respective donor were detected Although RDM alone cannot be used to draw conclusions about water uptake, our results suggest that *Hap-5B-RDMa* and *Hap-5B-RDMb* can increase transpiration in a genotype-dependent manner.

Chapter 4 aimed to investigate the effect of *Hap-5B-RDMa* and *Hap-5B-RDMb* on NUE, NupE, and NutE. Several studies have explored the impact of root-associated traits on NUE, including Foulkes et al. (2009), Kenobi et al. (2017), and Duncan et al. (2018). It is widely accepted that the main source of grain N is the N accumulated in plant biomass before anthesis. Therefore, genetic variation in grain yield and grain N content is primarily attributed to pre-anthesis N accumulation rather than post-anthesis N remobilization (Gaju et al., 2011). Under well-watered conditions, we found that NILs carrying *Hap-5B-RDMa* and *Hap-5B-RDMb* exhibited higher nitrogen derived from fertiliser (Ndff) than their respective recipients. This may indicate an increased NupE before anthesis. Various studies have investigated whether NupE or NutE is more effective in improving NUE in both hexaploid and tetraploid wheat. These studies have also described the genetic variation for NupE and NutE (van Sanford et al., 1986; Le Gouis et al., 2000; Barraclough et al., 2010; Gaju et al., 2011; Wang et al., 2011). A two-year experiment using three N levels reported that NupE explained 62-70% of the genetic variation for NUE

(Barraclough et al., 2010). Similar findings were reported in other studies, where NupE accounted for 54-63% of NUE (van Sanford et al., 1986; Le Gouis et al., 2000).

More recent studies demonstrate that the influence of NupE and NutE on NUE depends on soil N availability (Wang et al., 2011). This study did not find significant genetic-environmental interactions. NupE and NUE correlated significantly with each other under low and high N availability, while NutE showed significant effects on NUE only under high N availability (Le Gouis et al, 2000). NupE and NutE have been found to have varying degrees of influence on NUE at different nitrogen levels. Wang et al. (2011) assigned a higher importance to NupE due to its strong influence on NUE at several N levels. On the other hand, Barraclough et al. (2010) found interactions in NUE between different N levels, but a higher significance of NutE. This is consistent with studies that reported NutE largely describes the variation of NUE under low-N conditions (Gaju et al., 2011). The study found that for maize, genetic variation of NUE under high-N conditions was mainly described by NupE, whereas NUE variation was mainly described by NutE under low-N conditions (Gallais et al., 2004). In both experiments, genotypes carrying the haplotype blocks *Hap-5B-RDMa* and *Hap-5B-RDMb* showed positive effects on Ndff. Additionally, the results suggest that these haplotype blocks are particularly advantageous under well-watered conditions. Furthermore, the donor lines exhibited elevated Ndff values in drought scenario 2, where sufficient water was assumed to be available at 14 days after heading. Additionally, two out of three NILs demonstrated higher Ndff levels than their corresponding recipient lines under well-watered conditions. Thus, the QTL interactions with the genetic background of the recipient lines were observed for the well-watered variant in combination with Ndff, as one NIL shows lower Ndff than its parental line, similar to the relationship between *Hap-5B-RDMa* and *Hap-5B-RDMb* and the transpiration parameters.

5.3. Conclusion and future prospects

Due to climate change and the increasing frequency of droughts, it is crucial to identify and develop traits that enhance adaptation and improve crop yield stability in drought-prone regions with low rainfall. This thesis presented detailed insights and utilisation possibilities of modern phenotyping methods for future wheat and sorghum research and its relevance for breeding.

With regard to the EV trait in wheat, the key characteristics and its relationship with other traits, such as biomass, were successfully identified and have expanded our understanding of the EV trait. Additionally, this thesis presented effective methods for detecting EV both in the field and under controlled conditions. Notably, the connection between leaf length parameters and NDVI underscores index’s potential, especially with advancements in UAV (Shi et al., 2016, Di

Gennaro et al., 2018; Naser et al., 2020). However, potential interactions due to environmental factors must be clarified through practical crop management to enhance understanding, as factors like sowing time, depth, rate, and row spacing may influence EV. The interaction and value of EV in specific target environments also needs clarification. Yield predictions based on EV can be challenging since the trait is recorded at an early developmental stage and can be affected by numerous complex environmental factors later. For instance, abiotic stress during key developmental stages like tillering and flowering significantly impacts yield and its components. Nonetheless, EV is crucial for good crop establishment and can affect yield even at early stages.

Sorghum is already cultivated in arid regions due to its capacity to withstand drought stress. It is therefore of particular importance in sorghum breeding to gain insights into which growth stages the yield performance of sorghum is particularly at risk from drought events. Furthermore, in order to breed drought-stress-resistant varieties, it is necessary to precisely define and identify potential traits of interest. The results presented in this thesis offer new insights into sorghum's morphological and physiological drought response, as well as its effects on grain yield. However, further studies with a strong emphasis on biomarkers of stress and transcriptomic analyses are needed to provide further insights into drought adaptation strategies.

Optimized roots systems have a great potential to improve water uptake and NUE. The use of a precision phenotyping platform combined with ^{15}N tracer-based tracking of nitrogen uptake and remobilisation provides new insights into the effects of genetically altered root biomass on NUE and water uptake under varying water regimes. This thesis demonstrates the potential of modifying the genetic composition of the genetic composition of the locus on wheat chromosome 5B to reduce transpiration and improve nitrogen uptake. The results indicate that these effects are dependent on the genetic background of the recipient and water availability, demanding further research to explore these interactions. Firstly, the implementation of full growth-cycle experiments in high-resolution phenotyping platforms will provide more detailed information on N uptake and N translocation under different irrigation treatments, which may assist in the identification of suitable target environments for genotypes with enhanced root biomass. Secondly, although the design of the DroughtSpotter system enables a simultaneous and continuous examination of macro-physiological traits, it is recommended that the additional examination of micro-physiological traits, such as stomatal aperture, be conducted in order to obtain higher resolutions on trait characteristics and interactions. Secondly, field trials of the

NILs and their respective recipients will enable a more detailed evaluation of their performance in diverse environments. The initial results presented here suggest that the introgression of QTL conferring altered root biomass can be a rapid, useful and valid approach to improve NUE in wheat breeding programmes.

6. Summary

Wheat (*Triticum aestivum* L. *ssp. aestivum*) is one of the three most important crops globally, contributing about 21% to the world's food supply. Its domestication occurred in the Fertile Crescent. Wheat's evolution is based on allopolyploidization, resulting from hybridization with Aegilops species approximately one million years ago. Diploid and tetraploid species emerged first, followed by hexaploid wheat from the hybridization of tetraploid species and diploid wild grass. Sorghum (*Sorghum bicolor* L. Moench) is a crucial cereal, ranking fifth in global production from 2010 to 2020. Sorghum's domestication began in Northeast Africa, in present-day Ethiopia and Sudan, around 4000-3000 BC. Disruptive selection practices led to many improved Sorghum varieties, which spread through human migration and trade to other regions of Africa, India (around 1500-1000 BC), the Middle East (around 900-700 BC), China (around 400 AD), and eventually Europe (around 1204 AD).

Climate change is resulting in the emergence of erratic drought conditions in numerous growing regions. Furthermore, the excessive use of nitrogen fertilisers can have adverse environmental impacts. These include high consumption of fossil fuels and the release of carbon dioxide (CO_2) during the artificial synthesis of ammonia, as well as negatively influencing ecosystems due to leaching or volatile atmospheric emissions. As a consequence of both the increasing global population and the concomitant environmental alterations induced by climate change, there is an immediate necessity for the development of a sustainable agricultural model, which will ensure the production of sufficient food and simultaneously reduce the environmental impact of agricultural practices.

In the present thesis, an investigation on the physiological characteristics as well as a detailed analysis on morphological and physiological response on drought stress was performed in wheat and sorghum. Furthermore, this dissertation offers an in-depth validation of the root-growth associated haplotype blocks *Hap-5B-RDMa* and *Hap-5B-RDMb* with nitrogen uptake, nitrogen remobilisation as well as on water use.

In Sorghum, the use of a modern gravimetrical phenotyping platform, revealed the impact of early vegetative drought on morphological and physiological response, such as on plant development, biomass and flowering time.

In wheat an assessment of a low-cost phenotyping methodology in the field and under controlled conditions was performed. Normalised difference vegetation index in field as well as the digital image analysis under controlled conditions revealed, revealed a significant impact of the second leaf on the trait characteristics. Furthermore, genome-wide association studies were performed to identify genomic determination of EV, revealing 42 makers associated with

vegetation index and two makers associated with projected leaf area.
Additionally, the unique combination of a gravimetrical coupled with ^{15}N tracer-based tracking revealed for all varieties, carrying the root-growth associated trait, significant higher nitrogen uptake in the root organs, compared to those not carrying such QTL. Further, lower transpiration rates were found for certain genotype – background interaction.

In summary, in light of the changing climatic growing conditions and the objective of attaining more climate-neutral production systems, the development of new lines is imperative. These must be more resilient to abiotic stress factors such as drought stress, while still being able to adequately meet the demand for high-quality food under lower input factors. This dissertation provides a significant contribution to the understanding of early vegetative drought stress on morphological and physiological growth parameters in sorghum. In addition, this work presents a cost-effective method for phenotyping leaf area and novel insights into the genetic control of early vigour and nitrogen uptake in wheat. The findings presented here can be used as potential targets for breeding to develop more efficient varieties.

7. Zusammenfassung

Weizen (*Triticum aestivum* L. *ssp. aestivum*) ist eine der drei wichtigsten Nutzpflanzen weltweit und trägt etwa 21 % zur weltweiten Nahrungsmittelversorgung bei. Seine Domestizierung fand im Fruchtbaren Halbmond statt. Die Evolution des Weizens basiert auf Allopolyploidisierung, die vor etwa einer Million Jahren durch Hybridisierung mit *Aegilops*-Arten entstand. Zuerst entstanden diploide und tetraploide Arten, gefolgt von hexaploidem Weizen durch die Hybridisierung von tetraploiden Arten und diploidem Wildgras.

Sorghum (*Sorghum bicolor* L. Moench) ist ein bedeutendes Getreide und rangierte von 2010 bis 2020 weltweit an fünfter Stelle der Produktion. Die Domestizierung von Sorghum begann in Nordostafrika, im heutigen Äthiopien und Sudan, um 4000-3000 v. Chr. Disruptive Selektionspraktiken führten zur Entwicklung zahlreicher verbesserter Sorghum-Sorten, die sich durch menschliche Migration und Handelswege in andere Regionen Afrikas, nach Indien (ca. 1500-1000 v. Chr.), in den Nahen Osten (ca. 900-700 v. Chr.), nach China (ca. 400 n. Chr.) und schließlich nach Europa (ca. 1204 n. Chr.) ausbreiteten.

Der Klimawandel führt, in zahlreichen Anbauregionen, zu immer häufiger erratisch auftretenden Dürrebedingungen. Darüber hinaus kann der übermäßige Einsatz von Stickstoffdüngern negative Umweltauswirkungen haben. Diese umfassen einen hohen Verbrauch fossiler Brennstoffe und die Freisetzung von Kohlendioxid (CO_2) während der künstlichen Ammoniaksynthese sowie negative Einflüsse auf Ökosysteme durch Auswaschung oder volatile atmosphärische Emissionen. Aufgrund der wachsenden Weltbevölkerung und der damit einhergehenden Umweltveränderungen, die durch den Klimawandel hervorgerufen werden, besteht ein dringender Bedarf an der Entwicklung eines nachhaltigen landwirtschaftlichen Modells, das die Produktion ausreichender Nahrungsmittel sicherstellt und gleichzeitig die Umweltauswirkungen landwirtschaftlicher Praktiken verringert.

In der vorliegenden Dissertation wurde eine Untersuchung der physiologischen Merkmale von Eigenschaften im Zusammenhang mit der Trockenstressresistenz sowie eine detaillierte Analyse der morphologischen und physiologischen Reaktionen auf Trockenstress bei Weizen und Sorghum durchgeführt. Darüber hinaus bietet diese Dissertation eine eingehende Validierung der mit dem Wurzelwachstum assoziierten Haploblöcke *Hap-5B-RDMa* und *Hap-5B-RDMb* in Bezug auf Stickstoffaufnahme, Stickstoff-Remobilisierung und Wasserverbrauch.

Im Sorghum zeigte der Einsatz einer modernen gravimetrischen Phänotypisierungsplattform die enormen Auswirkungen von frühem vegetativen Trockenstress auf morphologische und physiologische Reaktionen, wie beispielsweise die Pflanzenentwicklung, Biomasse und Blütezeit.

Im Weizen wurde eine kostengünstige Phänotypisierungsmethode sowohl im Feld als auch unter kontrollierten Bedingungen bewertet. Der normalisierte Differenzvegetationsindex (NDVI) im Feld sowie die digitale Bildanalyse unter kontrollierten Bedingungen zeigten einen signifikanten Einfluss des zweiten Blattes auf die Merkmalscharakteristika. Darüber hinaus wurden genomweite Assoziationsstudien durchgeführt, um die genomische Bestimmung der frühen Vitalität zu identifizieren, wobei 42 Marker im Zusammenhang mit dem Vegetationsindex und zwei Marker im Zusammenhang mit der projizierten Blattfläche identifiziert wurden. Zusätzlich zeigte die einzigartige Kombination einer gravimetrischen Analyse mit der ^{15}N-Tracer-basierten Analyse bei allen Sorten, die das mit dem Wurzelwachstum assoziierte Merkmal tragen, eine signifikant höhere Stickstoffaufnahme in den Wurzelorganen im Vergleich zu denen, die dieses QTL nicht tragen. Darüber hinaus wurden niedrigere Transpirationsraten für bestimmte Genotyp-Hintergrund-Interaktionen festgestellt.

Zusammenfassend lässt sich sagen, dass angesichts der sich ändernden klimatischen Wachstumsbedingungen und des Ziels, klimaneutralere Produktionssysteme zu erreichen, die Entwicklung neuer Linien unerlässlich ist. Diese müssen widerstandsfähiger gegenüber abiotischen Stressfaktoren wie Trockenstress sein und dennoch in der Lage sein, die Nachfrage nach hochwertigen Lebensmitteln unter geringeren Inputfaktoren zu erfüllen. Diese Dissertation liefert einen wesentlichen Beitrag im Verständnis von frühen vegetativen Trockenstress auf morphologische und physiologische Wachstumsparameter bei Sorghum. Darüber hinaus stellt diese Arbeit eine kostengünstige Methode zur Phänotypisierung der Blattfläche vor, sowie neuartige Erkenntnisse zum genetischen Einfluss auf den Frühaufgang und der Stickstoffaufnahme beim Weizen. Die hier vorgestellten Erkenntnisse können als potenzielle Ziele für die Züchtung verwendet werden, um effizientere Sorten zu entwickeln.

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List of abbreviations

BCNAM	Backcross-nested association mapping population
DS1	Drought stress 1
DS1R	Drought stress 1 recovery
DS2	Drought stress 2
EV	Early vigour
Fv/Fm	Maximum quantum yield of photosystem II (PSII) in photosynthesis
GA	Gibberellic acid
GNDVI	Green normalised difference vegetation index
GWAS	Genome wide association study
L1	First leaf
L2	Second leaf
MAS	Marker-assisted selection
MR-NAM	Multi reference nested association mapping population
N	Nitrogen
NAM	Nested association mapping poulation
NDRE	Normalised difference red edge index
NDVI	Normalised difference vegetation index
NH_4^+	Ammonium
NIL	Near-isogenic Line
NO_2^-	Nitrite
NO_3^-	Nitrate
NUE	Nitrogen use efficiency
NupE	Nitrogen uptake efficiency
NutE	Nitrogen utilisation efficiency

QTL	Quantitative trait loci
RDM	Root dry mass
SLN	Specific leaf area per unit nitrogen
SNP	Single nucleotide polymorphism
UAV	Unmanned aerial vehicle
WUE	Water use efficiency
WW	Well-watered

Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice".

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